THE AMERICAN JOURNAL OF PATHOLOGY

VOLUME XIII NOVEMBER, 1937 NUMBER 6

THE PATHOGENESIS OF DIETARY NEPHRITIS IN THE RAT *

E. M. MEDLAR, M.D., AND N. R. BLATHERWICK, PH.D.

WITH THE ASSISTANCE OF JOHN M. CONNOLLY, M.D., PHOEBE J. BRADSHAW, AND SUSAN D. SAWYER, A.B.

(From the Hegeman Memorial Laboratory of the Sanatorium of the Metropolitan Life Insurance Company, Mt. McGregor, N. Y., and the Biochemical Laboratory of the Metropolitan Life Insurance Company, New York City)

Attempts to produce changes in the kidneys of experimental animals which would simulate the pathological alterations observed in diffuse nephritis in man have been made by a considerable number of investigators. A review of the literature on this subject is unnecessary since Horn [1] has recently published an exhaustive general review on experimental nephropathies. Suffice it to say that at present bacterial toxins and parenterally introduced proteins (nephrotoxins, and so on) are considered of greater etiological import in diffuse degenerative changes in the kidney than are dietary factors. Concerning the results obtained from feeding diets containing an excess of protein, Horn comments as follows: "Although tubular lesions may occur, the bulk of the experimental evidence seems to indicate that the degenerative alterations following such a regimen are minimal, and that the only anatomic result is a work-hypertrophy of the kidney consequent to the increased excretion of protein."

In a recent report [2] we have presented the results obtained from a rather extended series of experiments with rats on different dietary combinations. In Table I is presented a brief summary of some of our observations, especial reference being made to the percentage of protein in the various diets and to the number of rats in which the nephritis was of sufficient severity to be the primary cause of death.

* Received for publication July 6, 1937.

From the table it will be noted that the occurrence of nephritis varied widely with the first 6 diets and that female rats were more refractory than the males to its development. It is apparent that the amount of protein in the diet was not necessarily the determinant as to whether or not nephritis developed (compare Liver Diet XII and Casein Diet I). Another point not shown in the table was that rats with one kidney removed were more liable to develop

TABLE I

Summary of Previous Dietary Experiments with Special Reference to Percentage of Protein and to Extensive Nephritis

Type of diet	Protein	Male rats			Female rats		
		Number of animals	Average length of life	Number with extensive nephritis	Number of animals	Average length of life	Number with extensive nephritis
	%		*days*			*days*	
Stock II	23.3	19	567	0	32	551	0
Liver I	51.1	26	496	24 (92.3%)	14	523	8 (57%)
Liver XII	22.4	18	395	12 (66.6%)	16	511	5 (31.2%)
Casein I	72.1	6	676	2 (33.3%)	4	722	1 (25%)
Casein II	36.0	6	694	1 (16.6%)	6	631	0
2899, 2899A, 2899 & 2899A modified	25.0 to 27.5	28	563	0	26	563	0
Liver Diets XIV, XV & XX	51.1	..	..	..	18	386	17 (94.4%)

extensive nephritis more rapidly than intact rats. The difference between the male and female rats in their tendency to develop nephritis was thought possibly to be due to a smaller consumption of the diet by the females. Desiccated thyroid was added to Liver Diet I in different amounts with the idea that the metabolism would be increased and that the female rats would thus be induced to consume larger amounts of food. Female rats with one kidney removed were placed on such diets and the results obtained, as shown in Diets XIV, XV and XX in Table I, were comparable with those of the male rats on Liver Diet I. These experiments indicated that the thyroid of itself had some influence on the production of the nephritis since the food consumption was not enough

greater to account for the effects produced. Also, the amount of desiccated thyroid in the diet had a direct bearing on the length of time it took to develop a fatal nephritis. Three rats on a diet containing 0.4 per cent thyroid all developed extensive nephritis with an average length of life of 148 days, and 9 rats on a diet containing 0.1 per cent of thyroid all developed extensive nephritis with an average length of life of 539 days.

During the course of our previous experiments there was an unsuccessful attempt to determine the pathogenesis of the nephritis. Individual variations in the rats and the slowness of development of the nephritis with the diet used defeated the purpose of the experiment. Since Liver Diet XIV was found to induce extensive renal damage in a relatively short time in nephrectomized female rats, it was decided to utilize this diet in a study of the progressive phases of renal damage. The results of these experiments are herein presented.

Materials

Twenty-four young female rats from which the right kidney had been removed were fed Liver Diet XIV which had the following composition:

Beef liver (dried)	75.0 parts
Lard	15.0 "
Yeast (dried, Harris)	5.0 "
Cod liver oil (Squibb)	3.0 "
Salt mixture (Osborne & Mendel)	1.0 "
Calcium carbonate	0.6 "
Thyroid (desiccated, Armour)	0.4 "

The beef liver was carefully freed from its connective tissue and vessels, cut into small pieces, dried at a moderate temperature, passed through a chopper, dried again at a moderate temperature and then pulverized. Greens were added to the diet twice weekly.

As controls, 12 young female rats, having had a right nephrectomy, were fed Stock Diet II which had the following composition:

Wheat	55.0 parts
Klim (dried milk)	25.0 "
Beef muscle (dried)	12.0 "
Yeast (dried, Harris)	5.0 "
Sodium chloride	2.0 "
Calcium carbonate	1.0 "

The lean beef muscle was carefully freed from fat and connective tissue, passed through a chopper, dried at a moderate temperature and then pulverized. Greens were added to the diet twice weekly.

Stock Diet II was selected as the control diet since previous studies had shown that rats did not develop nephritis when maintained on it.

After being placed on the experimental diets the rats were killed at intervals in groups of three to determine the successive steps that led to the extensive renal changes. Throughout the experiment the animals were kept in separate cages in a room where temperature and air conditions were automatically controlled. A clean piece of blotting paper was placed in the pan of each cage daily except Sunday. Each rat had free access to food and water. Monthly examinations of urine were made to determine albumin values and the presence of casts. To obtain the urine the rats were placed in metabolism cages overnight without food. At the time of killing blood was obtained from each rat for chemical determinations.

Since none of the rats died of nephritis during the limited time set for the study, the tissues from rats on Liver Diet XIV in a previous experiment have been utilized to represent the terminal stages of the pathological process.

Technique

Albumin determinations on urine were made with the sulphosalicylic acid method. Chemical determinations on the blood were made by conventional methods.

The kidneys removed at nephrectomy and at autopsy were cut in two lengthwise, one part being preserved in Zenker's solution and the other in 10 per cent formalin. Paraffin sections were stained with hematoxylin and eosin, phloxine-methylene blue, or Mallory's aniline blue collagen stain without a counterstain. Frozen sections of formalin-fixed tissue were stained with Sudan III to demonstrate fat globules.

Results

Table II gives a compilation of age, weight, kidney weights and urinary findings for the experimental animals.

TABLE II

Comparative Data on Nephrectomized Female Rats Fed Stock Diet II and Liver Diet XIV

TABLE II

Comparative Data on Nephrectomized Female Rats Fed Stock Diet II and Liver Diet XIV

STOCK DIET II

Rat No.	Age at operation	Weight at operation	Weight of removed kidney (rt.)	Average weight	Days on diet	Weight at death	Weight of kidney (left)	Average kidney weight	Urinary findings (in months)										
									Albumin values *mg./100 cc.*						Presence of casts (pos. +; neg. o)				
									1	2	3	4	5	6	1	2	3	4	5
	days	*gm.*	*gm.*	*gm.*		*gm.*	*gm.*	*gm.*											
1438	50	140	0.78		33	185	1.23		50						o				
1439	52	120	0.67	0.61	33	145	0.75	0.88	10						o				
1440	52	105	0.39		33	130	0.65		o						o				
1441	52	125	0.60		62	172	0.92		..	..					o				
1448	37	80	0.36	0.45	58	150	0.85	0.90	..	o					+				
1449	37	85	0.40		58	155	0.93		..	20					o				
1550	30	75	0.42		90	185	0.96		..	20	20				o	o	o	o	
1551	30	70	0.46	0.42	90	205	1.03	1.01	10	30	20				o	o	o	o	
1552	30	70	0.37		90	235	1.05		10	10	20				o	o	o	o	
1556	28	65	0.37		148	210	0.98		10	10	10	20	20		o	o	o	o	o
1557	29	55	0.31	0.34	148	215	0.90	0.95	10	20	20	20	20		o	o	o	o	o
1558	29	60	0.34		148	205	0.96		10	10	10	20	20		o	o	o	o	o
Range	28–52	55–140	0.31–0.78			130–235	0.65–1.23		0–50	0–30	10–20								
Average	38	88	0.456			183	0.93		14	15	17	20	20		8.3% +	o	o	o	o

LIVER DIET XIV

1432	46	115	0.50		21	140	1.52		160						+				
1433	46	130	0.52	0.51	21	95	0.98	1.24	..						o				
1434	48	125	0.50		21	145	1.22		50						+				
1435	46	120	0.49		42	175	1.60		30	50					o	o			
1436	46	130	0.60	0.53	42	175	1.63	1.53	40	100					o	o			
1437	48	115	0.49		42	155	1.36		160	200					o	o			
1442	36	75	0.31		64	135	2.20		1400	400					+	+			
1443	36	85	0.37	0.34	64	175	1.90	2.00	500	400					o	+			
1444	36	80	0.33		64	155	1.90		600	800					+	+			
1445	37	90	0.38		90	195	2.12		20	100	400				o	o	+		
1446	37	85	0.34	0.36	90	185	2.77	2.40	250	600	400				+	+	+		
1447	37	80	0.36		90	175	2.30		250	750	1000				..	..	..		
1532	31	55	0.25		106	205	2.01		75	250	300	500			+	+	+	+	
1533	32	80	0.42	0.36	106	205	2.35	2.15	40	250	300	500			+	..	+	+	
1534	32	75	0.41		106	210	2.09		75	250	300	500			o	o	+	+	
1547	30	65	0.41		133	205	2.25		75	500	500	750			o	o	o	+	
1548	30	70	0.43	0.42	133	210	1.83	2.00	10	150	100	500			o	o	o	+	
1549	30	70	0.41		133	195	1.92		150	500	500	1000			o	o	+	+	
1554	31	75	0.43	0.40	155	235	3.54	3.13	100	200	500	1000	1500	1500	+	+	o	+	+
1555	41	75	0.37		155	215	2.72		40	50	200	500	800	700	+	o	o	+	+
1559	37	95	0.38		181	185	2.00		1000	2000	1500	50	100	750	+	o	+	..	..
1560	29	45	0.27	0.34	181	180	1.91	2.77	30	120	250	375	600	750	o	o	o	o	+
1561	29	60	0.36		181	230	4.40		30	200	500	750	1500	1000	+	o	+	+	+
Range	29–48	45–130	0.25–0.60			95–235	0.98–4.40		10–1400	50–2000	100–1500	50–1000	100–1500	700–1500					
Average	37	87	0.406			182	2.11		231	394	482	584	900	940	50% +	33% +	62% +	90% +	100%

The data on the two groups of rats are quite similar up to the kidney weights at autopsy and the urinary findings during the course of the experiment. It will be noted that in the Stock Diet II group the average kidney weight at autopsy was twice that of the

TABLE III

Chemical Determinations on Bloods of Rats Used in the Study of the Pathogenesis of Nephritis

Rat No.	Days	NPN	Total protein	Albumin	Globulin	Albumin / Globulin	Cholesterol
		mg./100 cc.	*gm./100 cc.*	*gm./100 cc.*	*gm./100 cc.*		*mg./100 cc.*
STOCK DIET II							
1438	33	37.5	5.21	3.06	2.15	1.40	68.3
1439	33	45.2	5.54	3.09	2.45	1.26	83.7
1440	33	44.1	5.62	3.14	2.48	1.27	..
1441	62	35.7	5.94	3.57	2.37	1.51	72.5
1448	58	33.9	5.47	3.61	1.86	1.94	..
1449	58	..	6.48	3.68	2.80	1.32	..
1551	90	35.5	5.83	3.00	2.83	1.06	60.7
1550	90	34.5	5.72	3.22	2.50	1.30	51.5
1552	90	No blood					
1556	148	39.5	5.65	3.24	2.41	1.34	76.9
1557	148	34.6	5.50	3.16	2.34	1.35	70.6
1558	148	35.2	5.67	3.39	2.28	1.49	83.7
LIVER DIET XIV							
1432	21	30.8	5.07	2.38	2.69	0.89	86.2
1433	21	49.8	5.37	2.76	2.61	1.06	..
1434	21	34.1	5.21	2.92	2.29	1.28	49.2
1435	42	37.6	5.55	3.18	2.37	1.34	88.9
1436	42	37.3	5.51	3.18	2.33	1.30	81.0
1437	42	34.7	4.84	2.69	2.15	1.25	57.4
1442	64	54.0	5.18	2.80	2.38	1.18	..
1443	64	41.7	4.95	2.76	2.19	1.26	67.8
1444	64	47.6	4.97	2.61	2.36	1.11	68.7
1445	90	24.2	5.07	2.42	2.65	0.91	59.2
1446	90	24.6	4.53	2.08	2.45	0.85	58.0
1447	90	32.6	5.31	2.17	3.14	0.70	76.0
1532	106	29.5	5.79	3.08	2.71	1.13	..
1533	106	29.2	4.94	2.52	2.42	1.04	63.5
1534	106	28.1	5.30	2.73	2.57	1.06	70.9
1547	133	38.5	6.01	3.55	2.46	1.44	108.0
1548	133	34.0	6.12	3.53	2.59	1.36	79.1
1549	133	42.3	5.80	2.89	2.91	0.99	108.6
1554	155	31.2	5.01	2.58	2.43	1.06	88.9
1555	155	33.7	4.88	2.61	2.27	1.15	65.8
1560	181	33.7	5.30	2.71	2.59	1.05	86.2
1561	181	36.8	4.97	2.51	2.46	1.02	96.1
1559	181	34.3	5.30	2.78	2.52	1.10	72.0

kidney removed surgically, whereas in the Liver Diet XIV group the average kidney weight at autopsy was five times that at nephrectomy. This represents in large part a true hyperplasia of renal tissue, as there was no evidence of cystic retention of urine either in the gross or on microscopic examination. The liver diet induced a hypertrophy at least twice that of the stock diet. That the hypertrophy was accompanied by degenerative changes in the kidneys from the rats on Liver Diet XIV is shown by the microphotographs which illustrate the article.

The urinary findings in the two groups are sufficiently different so that no comment is necessary. In our previous report [2] we have noted that the presence of casts and of abnormal albumin values signifies an altered kidney function but does not necessarily indicate that irreparable degenerative changes are occurring in the kidney. We have found, however, that casts and increased albumin output in the urine accompany consistently the pathological alterations that take place in the kidneys of rats on a diet of high liver content and that such a condition terminates in renal insufficiency with death therefrom.

In Table III the chemical determinations on blood are given for the two groups of rats. The data show no significant differences between the two groups. The absence of an increasing deviation from normal in the group on Liver Diet XIV in this experiment as the length of time on the diet increased would indicate that the renal impairment had not progressed far enough to cause retention. These findings agree with our previous observations [2] when we found that renal injury had to be very extensive before retention of nitrogenous products consistently occurred. There was urea retention in every one of 85 rats with a 4 plus nephritis (Figs. 24 and 25), whereas only 23 out of 39 rats with a 3 plus nephritis showed retention. In only 2 rats in our present series, Nos. 1560 and 1561, was the pathological damage severe, and even here it appears that the function of the kidneys at the time the animals were killed was sufficient to maintain fairly normal blood values. Neither of these animals presented lesions that would have been classed as a 3 plus or a 4 plus nephritis in our previous report. The process was not far enough advanced to justify such classification.

The kidney may be regarded as an aggregation of functional

units held together by a framework of reticulum and connective tissue in which the blood and lymph vessels are found. A functional unit consists of a tortuous, epithelial-lined tubule with a complex tuft of blood capillaries (glomerulus), capable of expansion and contraction, invaginated into the beginning of the tubule. The capillary tuft or glomerulus, being covered externally by epithelium, is the region where the blood comes in most intimate contact with the tubular epithelium and where at least the large portion of urinary filtration takes place. From its architecture it seems likely that the separation of substances from the circulating blood is brought about by the glomerular filtration plant and that the tubular epithelium serves mainly to work over, absorb, concentrate and excrete the glomerular filtrate. Considered in this light the functional unit should be considered as a whole since it is improbable that one portion can be severely damaged without the remainder being more or less involved. For simplicity of presentation we choose, however, to divide arbitrarily the functional unit into two parts and to discuss the glomerular and the tubular changes separately.

No abnormal glomeruli were observed in any of the kidneys removed surgically prior to the placing of the rats on the experimental diets. The glomeruli of the kidneys from the rats fed Stock Diet II appeared normal. It is possible that these structures were hypertrophied but of this we could not be certain.

The earliest definite glomerular changes in rats on Liver Diet XIV consisted of small foci in which there were more cells than normal. It seemed as if these foci were composed of cells of the blood or vascular system and that the epithelial cells were, if anything, fewer than normal. The earliest that such lesions were observed was at 60 days. In these early lesions small globules of fat were found on occasion. At this stage the major portion of the capillary bed appeared normal. Later the cells in the focal lesions became large and foamy and fat globules were usually present. As the disease progressed it was possible to find individual glomeruli with focal lesions of different size and appearance (Fig. 7), the latter suggesting a difference in age of the process. This progressive process led eventually to a partial (Fig. 2) or a complete (Fig. 12) sclerosis of the glomerulus.

Increase of the basement membrane (reticulum) of the capillary

tuft, as found in sections stained with aniline blue, was at first focal in character (Fig. 17), corresponding to the small focal lesions, such as seen in Figure 7. Later the reticular increase (Figs. 19, 20, 22 and 23) was found in half or more of the glomerulus. Adhesions between the capillary tuft and the capsular membrane were commonly seen. These adhesions caused a partial to complete obliteration of the capsular space. Increase of the reticulum of the capsular space, with considerable reduplication at times (Figs. 12 and 20), accompanied the increase of reticulum in the glomerulus.

A study of the successive changes in the glomerulus gave a definite impression that the structure became more cellular than normal at certain stages of the process. The increased cellularity appeared to be due to an increase of the epithelial cells covering the capillaries as well as an intravascular accumulation of monocytes. At no time was there found the intense inflammatory reaction (neutrophiles and fibrin) which is characteristic of certain acute nephritic lesions in man. No extravasation of blood into the capsular space was evident although on occasion the capillaries were found well filled with blood. At the end stage there were but few cells of any type in the sclerosed glomerulus (Fig. 12). The sclerosing process did not completely obstruct the capillary bed of the glomerulus for even the extensively sclerosed units showed a certain amount of blood within the capillaries.

The afferent and efferent vessels of the glomeruli (Figs. 22 and 23) were not involved in the process taking place within the capillary bed. In other words, the degenerative renal lesions were not dependent on vascular changes, unless those alterations are limited strictly to changes within the glomeruli.

The tubular epithelium and basement membrane appeared normal in all of the kidneys that were removed at operation prior to placing the rats on the experimental diets. No changes of undoubted significance were observed in the tubules of the kidneys from the group of rats fed Stock Diet II.

Significant changes in the epithelium and the basement membrane of the beginning of the tubules (capsules of the glomeruli) were not present in all tubules but they were sufficiently frequent in the group fed Liver Diet XIV, especially in the later stages, to suggest that they were an essential part of the pathological pic-

ture. It seemed as if the primary lesion was an injury to the epithelial cells which in some instances was severe enough to cause necrosis of individual cells. Following this injury there occurred a variable degree of epithelial hyperplasia with more or less conspicuous epithelial crescents (Figs. 10 and 11) being produced at times. These epithelial crescents were suggestive of similar structures present in some cases of human nephritis. Some of the crescents presented the appearance of a syncytium while in others individual cell borders could be distinguished. Thickening of the basement membrane was common in this portion of the tubules. This change seemed to be dependent on injury of the epithelial cells but was not restricted to the areas where epithelial hyperplasia occurred. The thickening of the basement membrane took the form either of broad bands or of splitting (or reduplication) of narrow bands. On occasion (Fig. 20) the split or reduplicated fibers extended in between the epithelial cells of a crescent. These epithelial and basement membrane changes were usually strictly limited to the capsular area of the tubule. On rare occasions similar changes were noted at the beginning of the proximal convoluted tubule (Fig. 19).

Degenerative changes in the proximal convoluted tubules could not be demonstrated. At an early stage the cells appeared to be larger and the granular structure seemed to be more conspicuous than normal. No evidence of necrosis or hyperplasia of the epithelial cells was found. Frequently the lumens of the tubules contained more granular and amorphous material than normal. In the end stages these tubules were often considerably distended with retained glomerular filtrate which in some instances, though not usually, assumed the appearance of casts. On account of the distention, the epithelium often had the appearance of a flattened cuboidal type. On occasion the cells contained brownish pigment which did not give a Prussian blue reaction and was suggestive of urochrome. More than a slight thickening of the basement membrane was rarely found. The increase of the basement membrane when present was possibly a reparative response to the stretching caused by the distention of the tubules.

Changes noted in the loops of Henle and the distal convoluted tubule will be considered together for all of this portion of the functional unit seemed to be simultaneously and equally involved.

At the start there was injury, which sometimes led to necrosis of individual cells (Fig. 18), followed by varying degrees of hyperplasia of the epithelial cells (Figs. 3, 4, 5, 6 and 9). Frequently the hyperplasia was so great that the tubules became packed with epithelial cells (Fig. 21). The appearance of the cells varied considerably. Some were filled with a brownish pigment, suggestive of urochrome; others were replete with large hyaline droplets; and many of the cells contained fat-staining droplets which varied in size and in numbers. Eventually a dissolution of the cells in this region of the tubule occurred with obliteration of the tubular structure.

Changes in the basement membrane (Figs. 18 and 21) accompanied the injury, hyperplasia and dissolution of the tubular epithelium. At first the thickening tended to be focal but later it became general. The increase might appear either as broad bands or as a reduplication of fibers which at times extended in between the hyperplastic epithelium and eventually obliterated the lumen of the tubule.

These tubular changes resulted in areas of fibrosis (Figs. 13 and 14) in which tubular structures had largely disappeared and in which a varying amount of lymphocytic and monocytic infiltration was commonly found (Figs. 12, 13 and 14). These fibrotic areas tended to prevent the outflow of urine and as a result cystic dilatation of the proximal convoluted tubules and capsular spaces of even the remaining normal functional units (Fig. 8) was produced. Such urine retention often caused considerable enlargement of the kidney (Fig. 24), presenting numerous cysts on gross examination.

The only abnormalities noted in the collecting tubules in these kidneys were evidenced in considerable numbers of casts in the late stages of the disease.

All kidneys, even though very extensively involved, have shown some normal functional units. Why some units escape the degenerative changes observed in others is but a matter of conjecture. The pathological process we have discussed above seems to be progressive in nature and it is conceivable that, could the animals survive, all functional units would eventually become involved.

Discussion

The use of experimental animals provides a method of study of many pathological processes which is superior to material obtained for study from similar lesions in human tissue. Carefully controlled procedures, repetition of experiments and the inclusion of relatively large numbers of animals help to rule out factors which are irrelevant but which from their apparent association with the disease in man may seem to bear a direct and important relation to the pathology encountered. Also it is possible with experimental animals to determine whether the pathological process depends on a single, therefore specific, factor or whether different factors may induce a similar process which thus would suggest non-specificity. There is, however, one thing that must always be borne in mind relative to results obtained from using experimental animals. The biological phenomena of the rat, for example, and of man are not identical. It is quite possible, therefore, that the factors which initiate a pathological process in the rat may differ from those in man and still the pathological picture may be very similar, if not identical, in both instances. In our consideration of the pathogenesis of dietary nephritis in the rat, we are not especially concerned as to the possibility or the probability that the initiating factors for the chronic nephritides in man and in the rat are identical. Since we have been able,[2] under carefully controlled conditions, to bring about consistently a progressive degenerative change in the rat kidney which in the end simulates in many ways the final stage of chronic nephritis in man, we do consider it important to try to evaluate the series of events that take place within the kidney tissue.

It has been our experience that when a group of rats is placed under as similar conditions as is experimentally possible, some of the animals develop nephritis much more slowly than others. These individual variations make it impossible to give a precise chronology to the alterations observed in the kidney tissue. Rather it is possible to discuss only a series of events which seem to dovetail and which in the end lead to a functional and histological destruction of at least portions of functional units.

From the tissues we have studied it appears that the primal renal lesion is a damage to the filter bed of the glomerulus. In just

what way this structure is first affected, or in which stratum, is not clear. Eventually the endothelium, epithelium, and basement membrane are all involved. The primary damage seems to be focal in nature with progressive focal lesions developing, thus presenting lesions of different ages in a single glomerulus. In time the large majority of the glomeruli become sclerosed.

Since abnormal excretion of albumin and casts appears in the urine prior to any demonstrable renal lesions it seems reasonable to assume that the changes found in the tubules result from the presence of substances that have passed through the altered glomerular filter bed. A further substantiation of such a hypothesis is that focal areas of necrosis and hyperplasia of the epithelial cells and of increase of the basement membrane occur in portions of the tubule where there is no intimate contact between intertubular vessels and the tubule structure.

The early injury to the tubular epithelium seldom leads to a necrosis of more than a few individual cells. In the main the injury is sufficient to cause a considerable hyperplasia of the epithelium. This phenomenon is followed later by necrosis and dissolution of the hyperplastic tissue. The whole phenomenon may be regarded as an overwork of the epithelium. The process is selective in type since the proximal convoluted portion of the tubule appears to be little damaged. Richards and Walker [3] have shown that different regions of tubular epithelium perform different functions. It is possible then to attribute the changes noted in the tubular epithelium to a functional selectivity.

The changes that occur in the basement membrane of the tubules depend apparently on the escape through the epithelial covering of substances that cause a considerable increase in the amount of basement membrane material. The process is progressive in nature in the areas of the tubule in which the epithelium is involved, namely the glomerular capsule, the loops of Henle and the distal convoluted tubule. To a considerable degree the amount of distortion of the kidney architecture in the late phases of the disease is related directly to the extent and degree of increase of the basement membrane material. This apparently is the cause of the interstitial fibrosis so evident in extensively damaged kidneys.

In none of the kidneys have we encountered an acute inflammation. In the later stages of the disease lymphocytic infiltration of

the interstitial tissue was a common occurrence and in some instances was pronounced. This we believe is a reaction to damaged tissue and is non-infectious in nature. Including all of our dietary experiments with rats we have not found that spontaneous infections, which have at times been quite common, played a significant rôle in the production of nephritis.

This nephritis, related to certain diets, is a progressive degenerative disease. It appears to begin as a focal damage in the glomerular filter bed and is followed by a selective injury, hyperplasia, overwork and death of the tubular epithelium. Sclerosis of glomeruli and capsules and interstitial fibrosis in the region of the loops of Henle and the distal convoluted tubules represent the reparative end-stage of a burned-out process. The final picture simulates the so-called atherosclerotic nephritis in man with the arteries or arterioles remaining normal.

Smadel [4] has recently reported on the chronic phase of the nephritis produced in rats by "nephro-toxins." It has been the privilege of one of us (E. M. M.) to study sections from some of his specimens. The histological picture presented in Smadel's and in our rats in the chronic phase of the disease is very similar. The difference between the nephritis in the two sets of experiments lies in the fact that we have never encountered the early acute glomerular lesions which Smadel [4] reported. The provocative agent in Smadel's experiments and in our own would appear to be quite dissimilar. It is significant that the end results, in so far as the kidney lesions are concerned, are so similar.

It seems probable that the etiological factors capable of causing progressive degenerative nephritis are multiple and they may well be quite diverse in character. It is also conceivable that the final pathological picture is the same regardless of the nature of the provocative agent. We venture the suggestion that progressive degenerative nephritis hinges on the production of irreparable damage to the filter bed of the glomerulus. This damage may be initiated by toxic products produced during the course of an infection, by so-called nephrotoxins, by unknown abnormal metabolic products or by certain diets (at least experimentally). The sclerosing of glomerular tufts, the obliteration of capsular spaces, the ultimate degeneration of tubular epithelium, the interstitial fibrosis, the inflammatory reaction and the cystic retention probably follow

in the wake of the damaged filter bed. In the progressive degenerative nephritides the chronicity of the disease may well depend on the extent and the severity of damage to the filter apparatus.

Summary and Conclusions

We have previously shown that when rats are fed certain diets of high animal protein content progressive chronic degenerative nephritis can be produced consistently. The pathogenesis of this type of nephritis is here considered. It appears that the initial lesions are focal injuries in the filter beds of the glomeruli and that these are progressive in character. Subsequent to the glomerular damage, injury, hyperplasia and dissolution of the tubular epithelium of the glomerular capsule, loops of Henle and distal convoluted tubule occur. The end-result is a chronic degenerative nephritis in which the principal features are: sclerosis of glomeruli with or without obliteration of the capsular spaces; interstitial fibrosis in the regions of the tubule where the epithelium has been seriously affected; chronic inflammation, which may be considerable; and cystic dilatation of the proximal convoluted tubules, which may or may not be extensive.

The possible relation of experimentally produced dietary nephritis to the chronic nephritides in general is briefly discussed. A suggestion is ventured that chronic degenerative nephritis in general may depend primarily on an irreparable damage to the filter bed of the kidney and that the etiological factors initiating this primal damage may be multiple and diverse in character. We are of the opinion that it is inadvisable to attempt to designate the origin or nature of the etiological agents that induce progressive degenerative changes in the kidney, since information pertaining to the complex phenomenon is so incomplete.

References

1. Horn, H. The experimental nephropathies. *Arch. Path.*, 1937, **23**, 71.
2. Blatherwick, N. R., and Medlar, E. M. Chronic nephritis in rats fed high protein diets. *Arch. Int. Med.*, 1937, **59**, 572–596.
3. Richards, A. N., and Walker, Arthur M. Urine formation in the amphibian kidney. *Am. J. M. Sc.*, 1935, **190**, 727–746.
4. Smadel, Joseph E. Experimental nephritis in rats induced by injection of anti-kidney serum. III. Pathological studies of the acute and chronic disease. *J. Exper. Med.*, 1937, **65**, 541–555.

DESCRIPTION OF PLATES

Figures 1–14 and 24–26 are from sections stained with phloxine-methylene blue. Figures 15–23 are from sections stained with Mallory's aniline blue collagen stain without counterstain.

PLATE 124

FIG. 1. From the kidney cortex of a rat that had been on Stock Diet II 148 days. The histological picture is essentially normal. × 500.

FIG. 2. From the kidney cortex of a rat on Liver Diet XIV for 120 days. Note the focal glomerular lesion with large foamy cells. Note also the granular material within the capsular space and the proximal tubule. The presence of this granular material we interpret as an indication of damage to the glomerular filter bed. × 500.

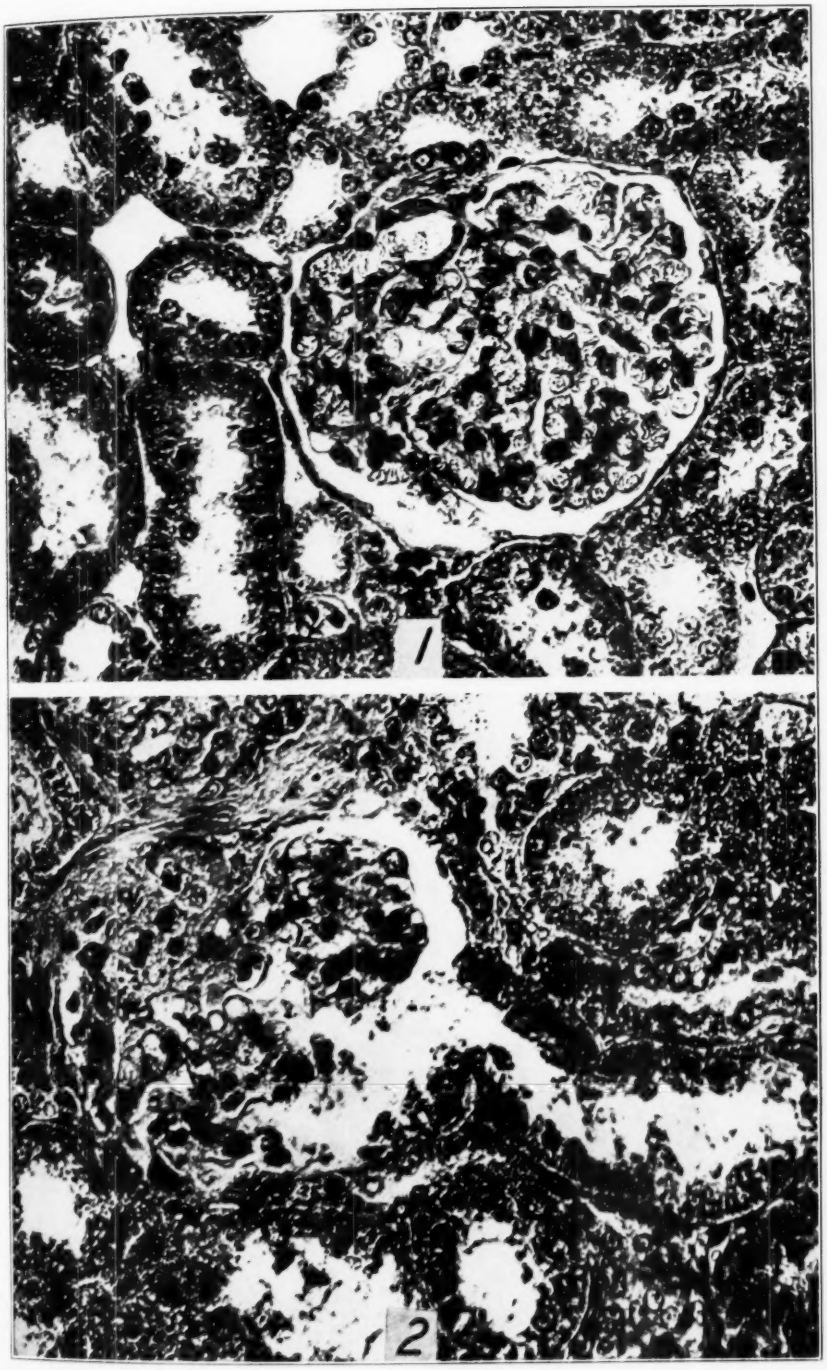


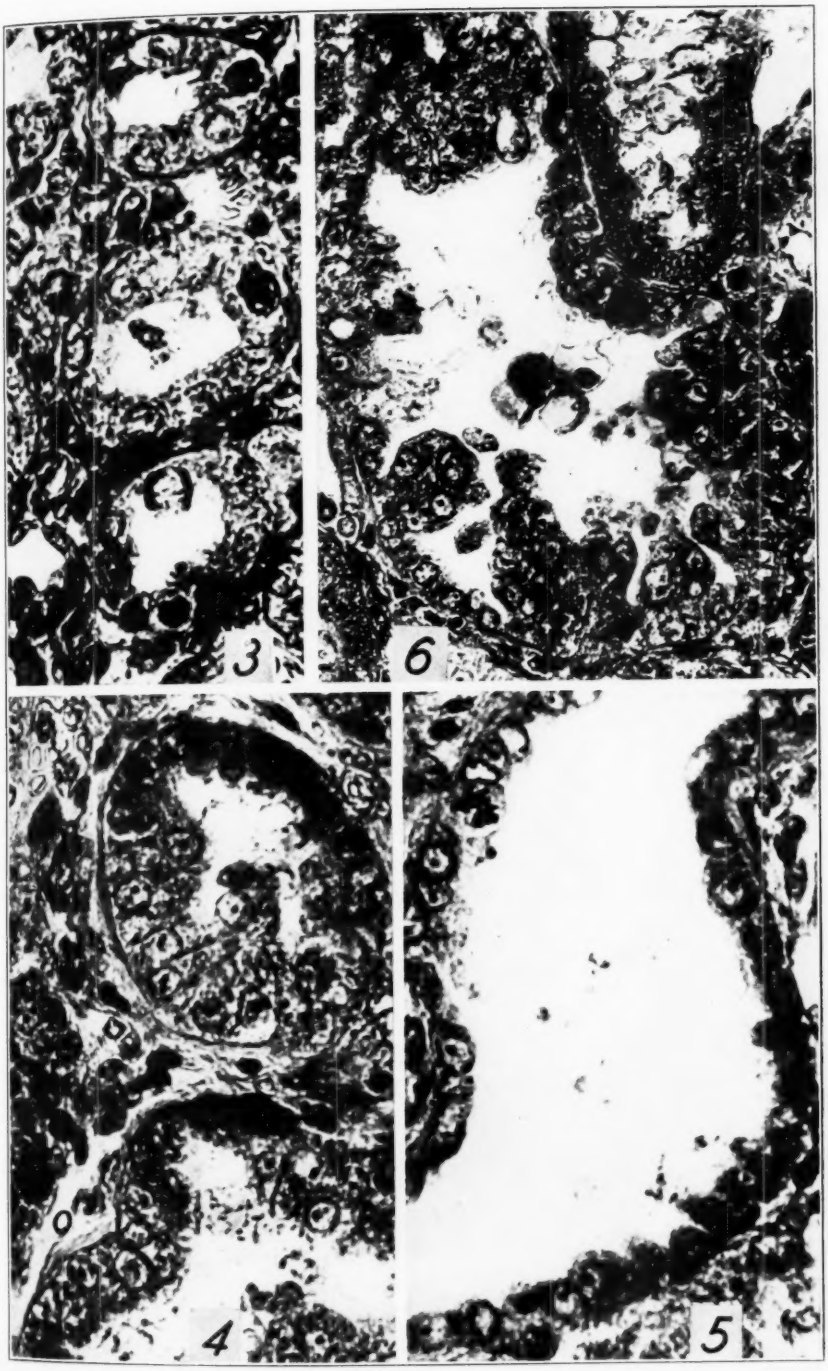
PLATE 125

FIG. 3. From the striate zone of the kidney from a rat on Liver Diet XIV for 60 days. Note the mitosis in an epithelial cell of a loop of Henle. × 800.

FIG. 4. From the striate zone of the kidney from a rat on Liver Diet XIV for 120 days. Note the considerable hyperplasia of the epithelium in a loop of Henle. The original epithelial cells form a single layer in the upper quadrant. × 800.

FIG. 5. From a distal convoluted tubule of a rat on Liver Diet XIV for 90 days. Note mitotic figure in upper left hand quadrant. × 800.

FIG. 6. From a distal convoluted tubule of a rat on Liver Diet XIV for 120 days. The hyperplasing epithelium is taking the form of papillomatous projections. A mitosis is present in the group of cells in the upper portion of the tubule. × 500.

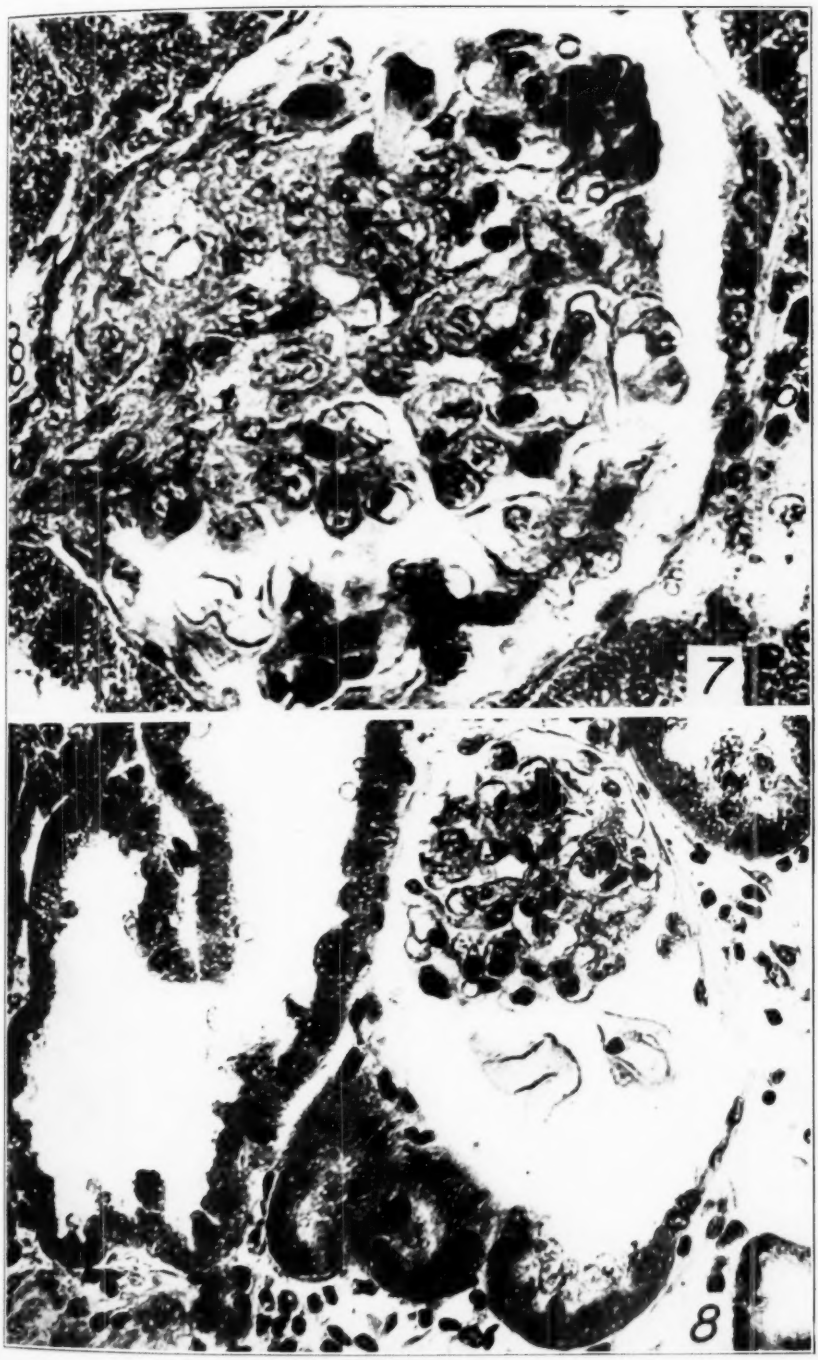


Medlar and Blatherwick Dietary Nephritis in the Rat

PLATE 126

FIG. 7. A glomerulus from a rat on Liver Diet XIV for 90 days. There are three focal lesions: left center, lower center and upper right border. The older lesion is composed of large foamy cells (fat-containing), probably monocytes. Focal lesions of different ages were often observed. Note the early crescent of capsular epithelium to the right. × 1000.

FIG. 8. A fairly normal functional unit from a rat on Liver Diet XIV for 10 months. In this particular kidney such units were scarce. The dilatation of the capsular space and proximal convoluted tubule is caused by interstitial fibrosis in the striate zone of the kidney. × 500.



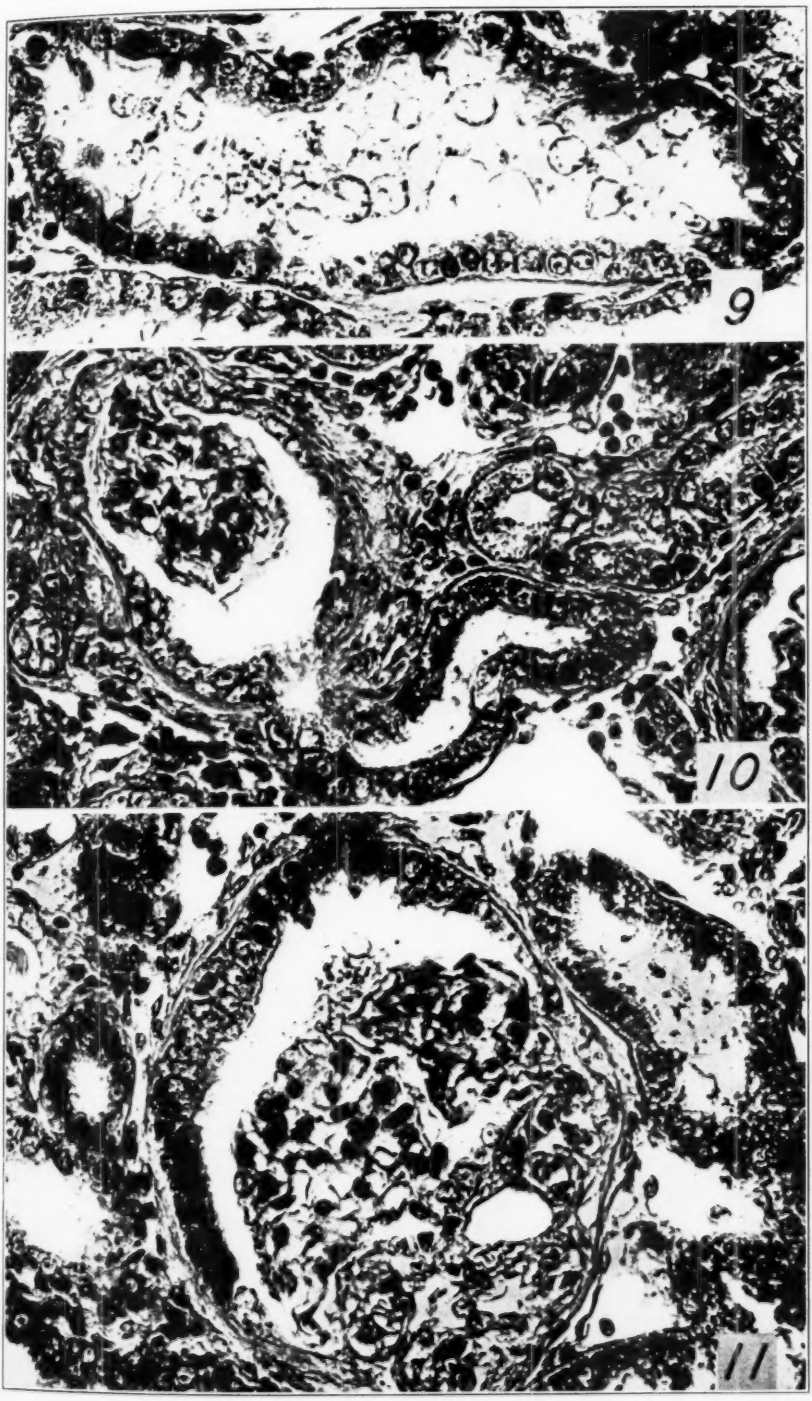
Medlar and Blatherwick Dietary Nephritis in the Rat

PLATE 127

FIG. 9. Tangential section of loop of Henle from a rat on Liver Diet XIV for 90 days. Note focal regenerated epithelium and thickened basement membrane along lower portion of tubule. This lesion is not in the proximity of intertubular vessels which are to the extreme left. The rest of the tubular epithelium has a frayed-out appearance. Note also that the tubule is filled with amorphous material which probably is glomerular filtrate. × 500.

FIG. 10. Glomerulus from a rat on Liver Diet XIV for 180 days. Note especially the crescent of capsular epithelium and the thickened basement membrane. × 500.

FIG. 11. Glomerulus from a rat on Liver Diet XIV for 150 days. The lower half of the glomerular tuft is pretty well sclerosed. A typical crescent is present. × 800.



Medlar and Blatherwick Dietary Nephritis in the Rat

PLATE 128

FIG. 12. A sclerosed glomerulus with obliteration of the capsular space from a rat on Liver Diet XIV for 208 days (from kidney shown in Fig. 25). Note the lymphocytic infiltration in interstitial tissue adjacent to glomerulus. The infiltration in this illustration is mild when compared to that of other areas of the same kidney. In this particular kidney uninvolved glomeruli were few in number. × 500.

FIG. 13. An area in the striate zone of the same section from which Figure 12 was taken. Note fibrosis, lymphocytic infiltration and lack of tubular structure. × 500.

FIG. 14. Another area in the striate zone from the same section from which Figures 12 and 13 were taken. Here the lesion is evidently an end stage with the fibrosis being the principal feature. The central portion of the picture suggests a fibrosed tubule similar to one shown in Figure 6 with two or three single epithelial cells remaining. × 500.

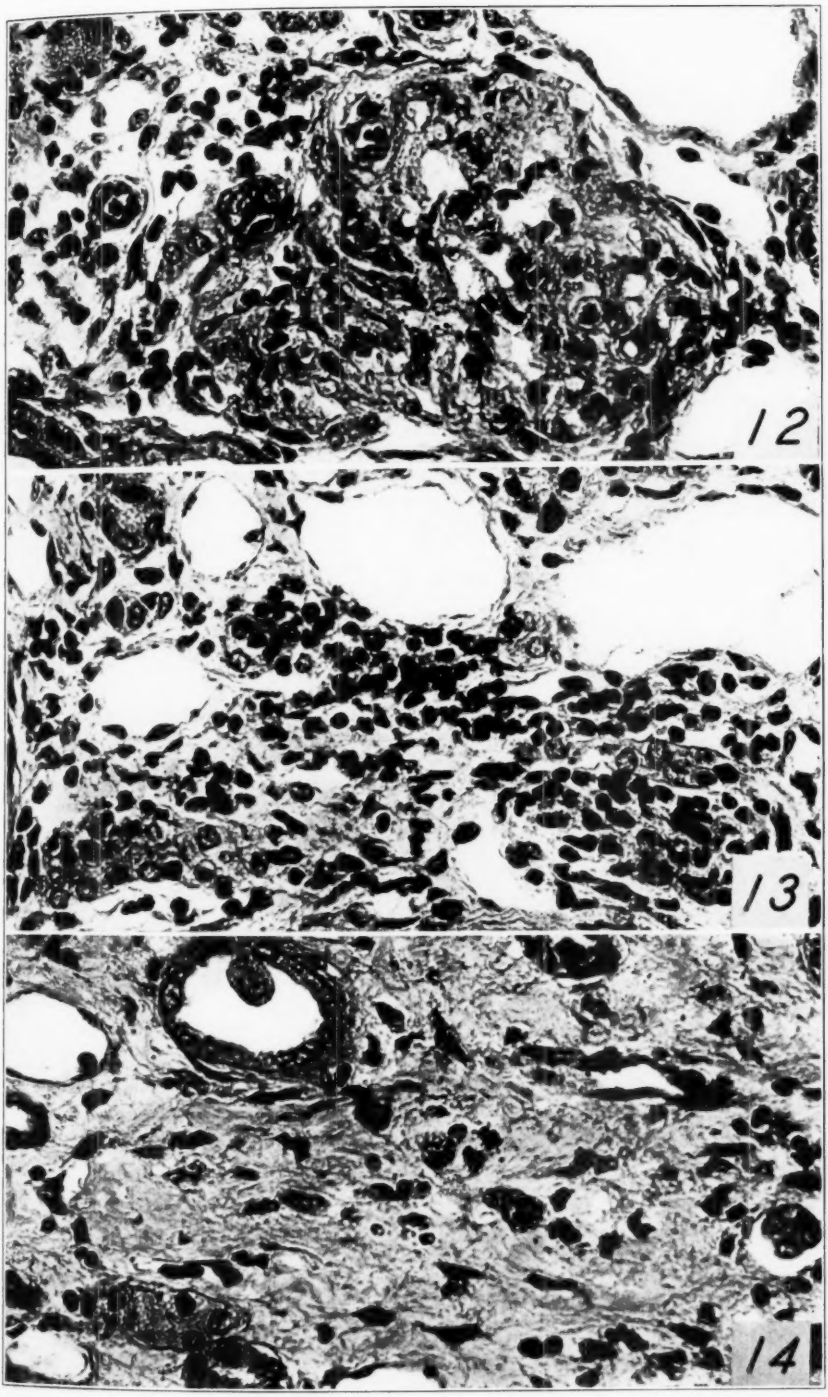
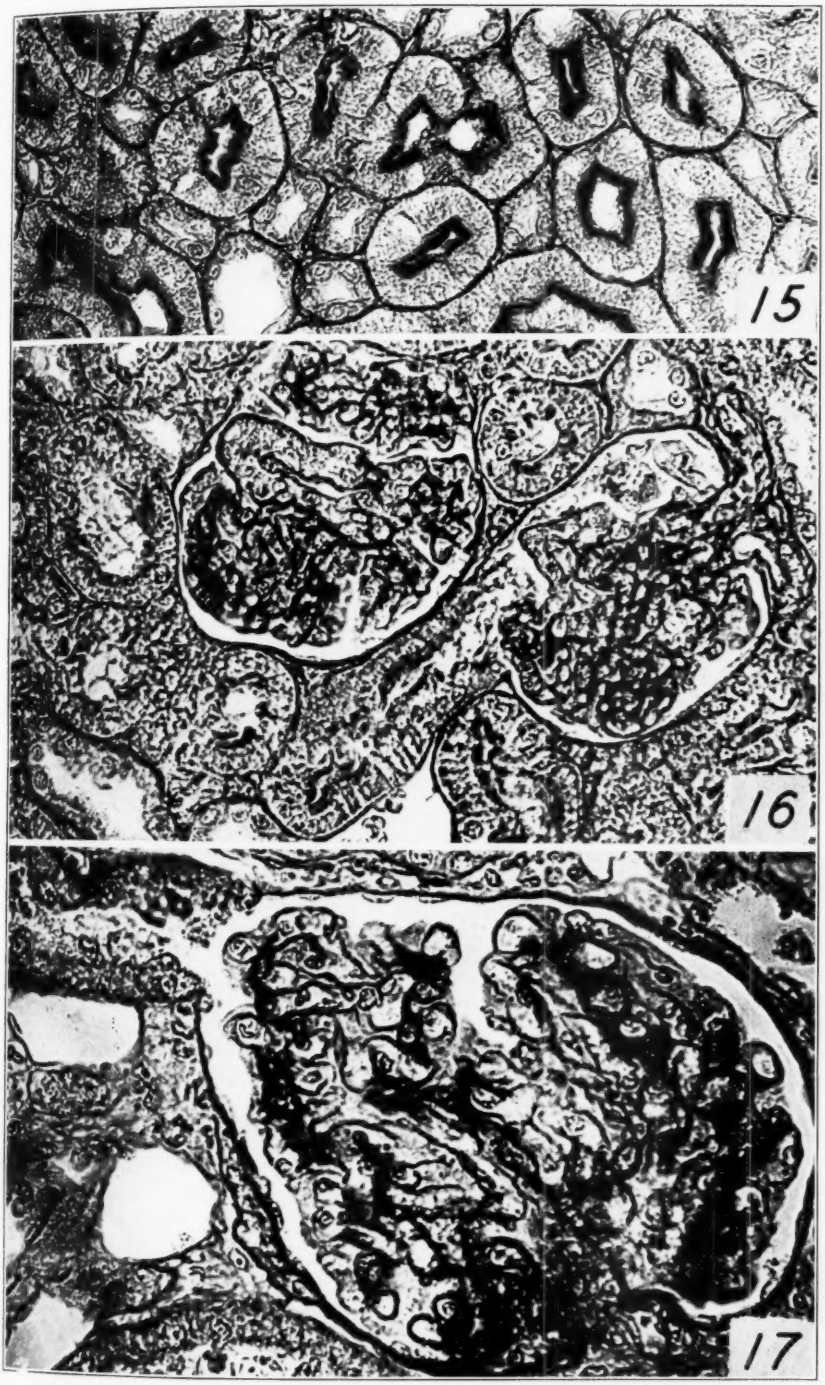


PLATE 129

FIG. 15. From the striate zone of a kidney from a rat on Stock Diet II for 148 days. This shows the normal basement membrane (reticulum) pattern. × 500.

FIG. 16. From the cortical zone of the same kidney section from which Figure 15 was taken. This shows the normal reticulum pattern for glomeruli and tubules in this region. × 500.

FIG. 17. Glomerulus from a rat on Liver Diet XIV for 60 days. Note the small focal thickening of the reticulum. × 500.



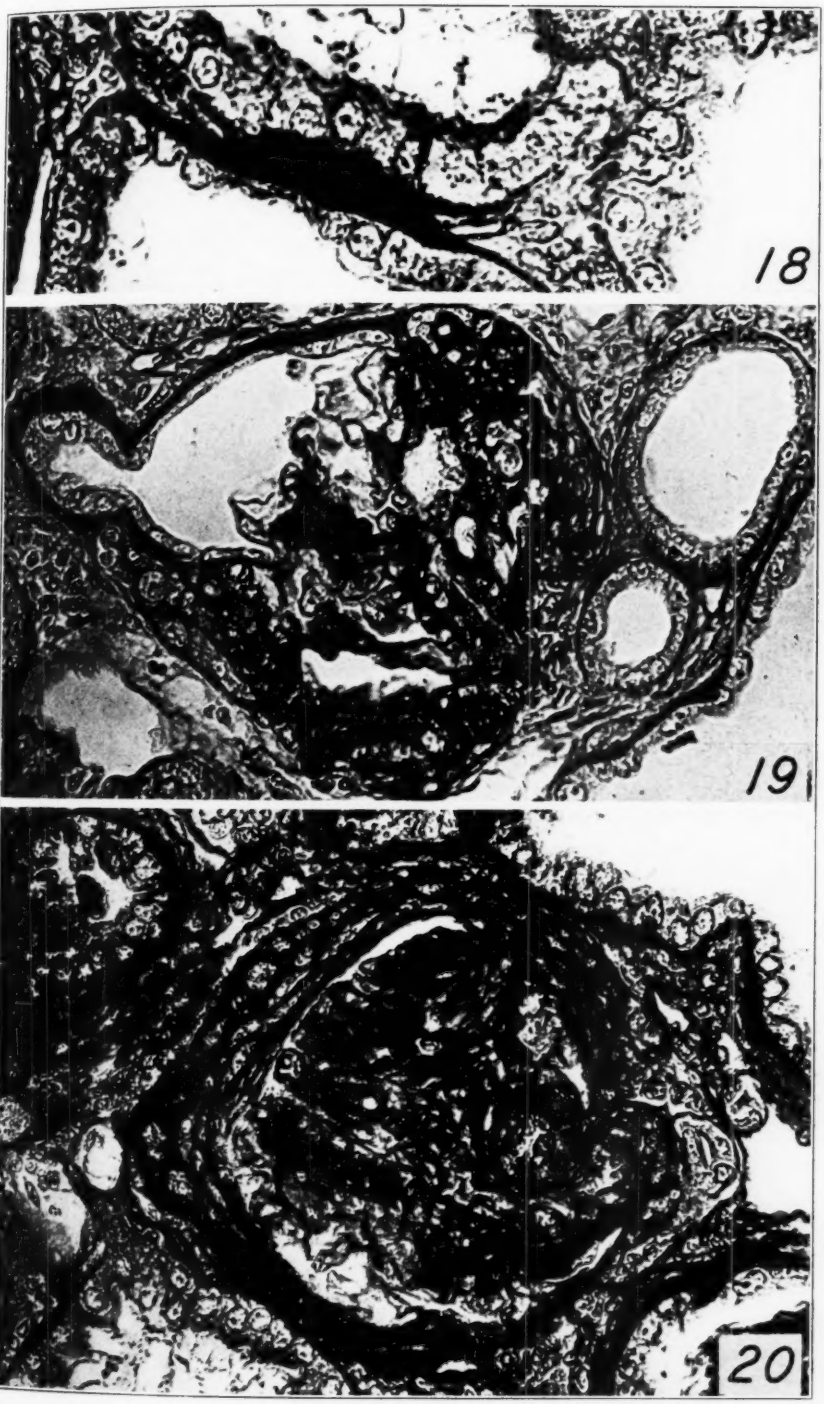
Medlar and Blatherwick Dietary Nephritis in the Rat

Plate 130

Fig. 18. From the same kidney from which Figure 9 was taken. On Liver Diet XIV for 90 days. Note focal thickening of basement membrane and two necrosed cells just below the thickened area. Such lesions are frequently found and seem to depend on material escaping from the tubule. Note also that the intertubular vessels lie to the right and left and are not in juxtaposition with the thickened membrane. × 800.

Fig. 19. From the same kidney as Figure 10. On Liver Diet XIV for 180 days. Note focal increase of reticulum in the tuft, the partial obliteration of the capsular space and the thickened membrane of the capsule which involves the beginning of the convoluted tubule. This latter lesion is not commonly seen. × 500.

Fig. 20. Glomerulus from a rat on Liver Diet XIV for 180 days. Note especially the reduplicated fibers of the capsular basement membrane which to the left extends between the epithelial cells of a crescent. Although the sclerosis is pronounced the capsular space is only partially obliterated. × 500.



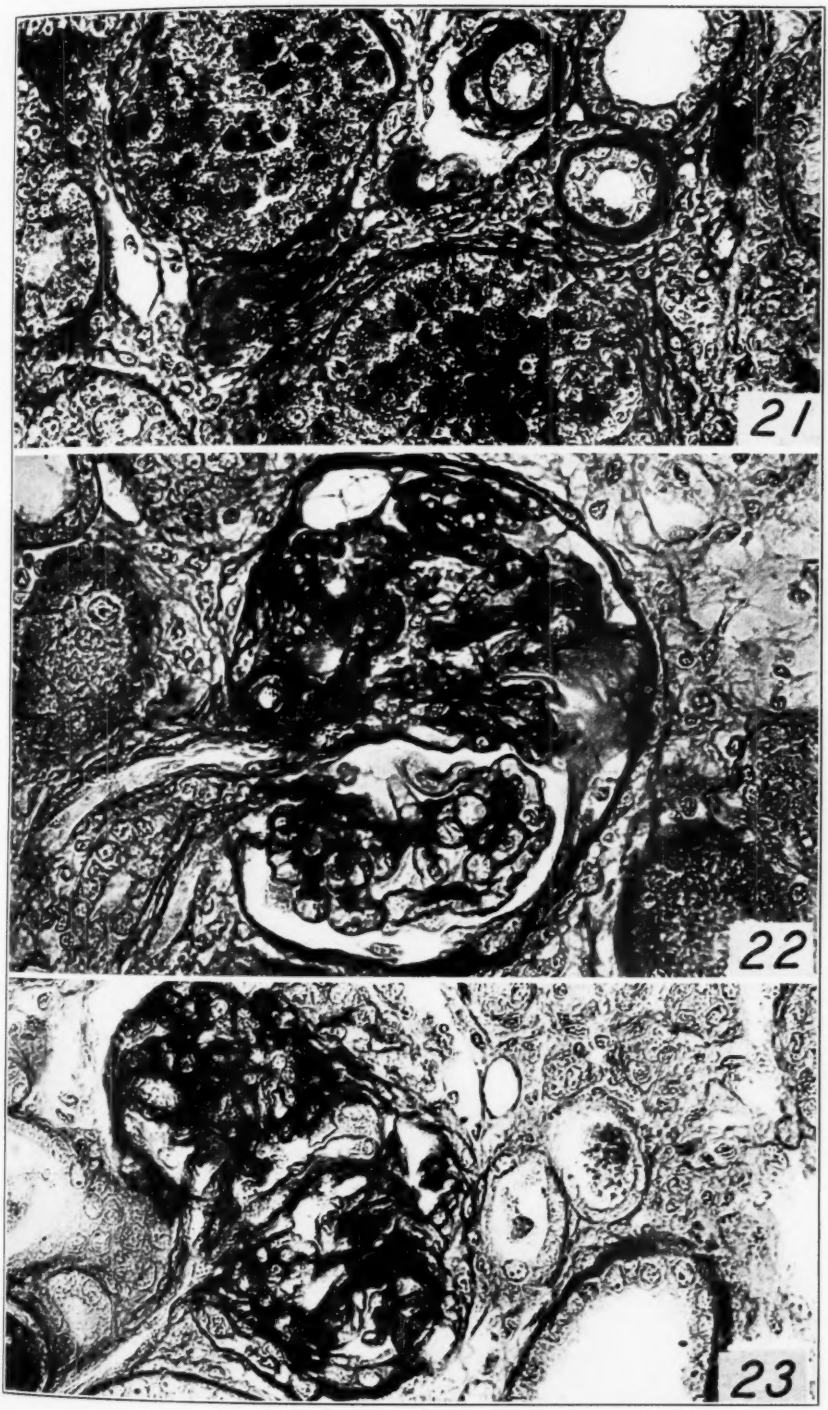
Medlar and Blatherwick Dietary Nephritis in the Rat

Plate 131

Fig. 21. From the striate zone of a kidney from a rat on Liver Diet XIV for 150 days. Tubules are filled with epithelial cells, many of which are disintegrating. The heavily stained cells are full of pigment. Around the larger loops of Henle the basement membrane fibers tend to be reduplicated while around the thin portions of the loop the basement membrane tends to remain in a much broadened band. Compare with the basement membrane pattern shown in Figure 15. × 500.

Fig. 22. A glomerulus from the same section from which Figure 20 was taken. Note focal thickening of reticulum in the tuft and the "adhesion" extending from the tuft to the capsular basement membrane. Note also that the afferent and efferent vessels to the left are normal. × 500.

Fig. 23. A sclerosed glomerulus from the same kidney from which Figure 12 was taken. Note especially that the vessel outside of the glomerulus is normal. In none of the kidneys have there been sclerotic changes in the arteries or arterioles. The pathology is confined primarily to the capillary tufts and the tubules. × 500.



Medlar and Blatherwick Dietary Nephritis in the Rat

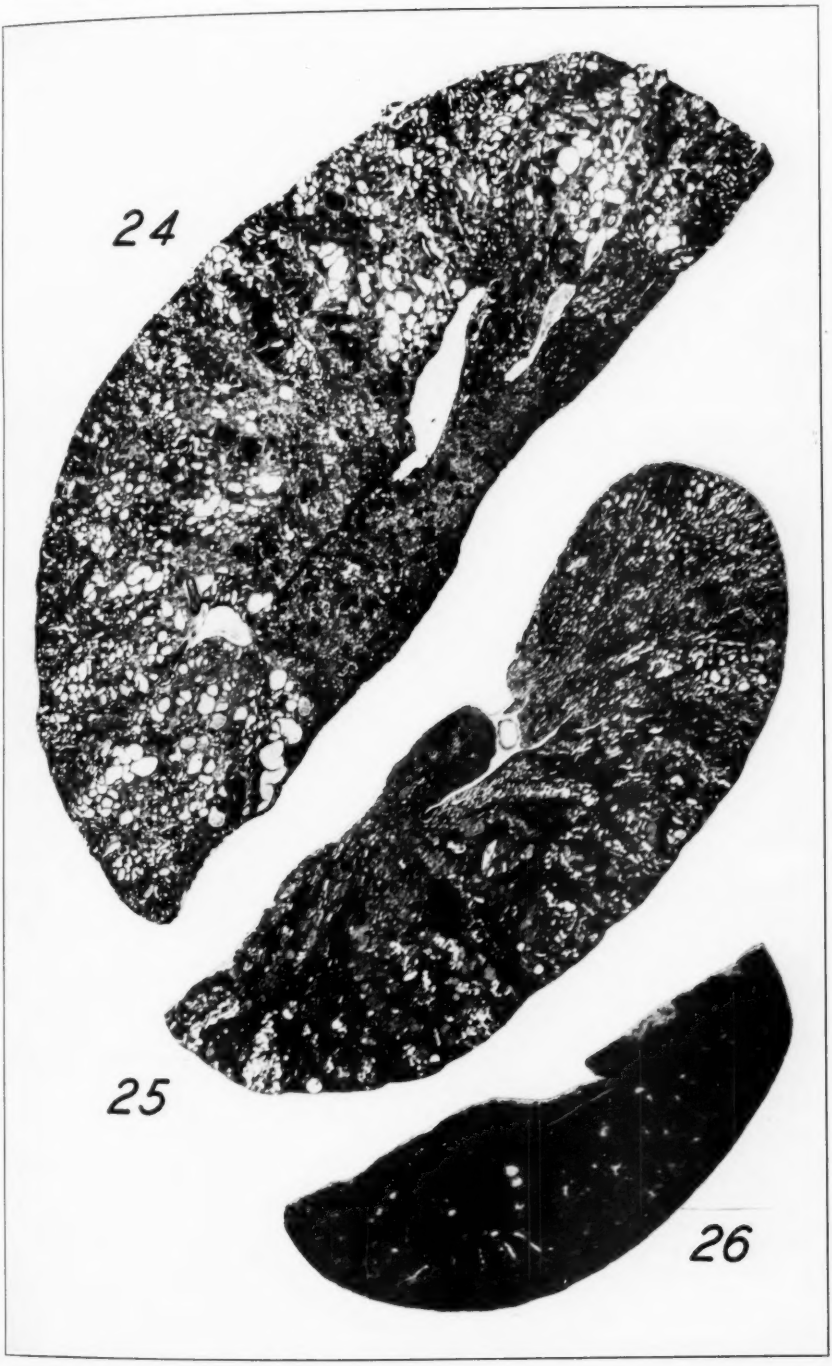
PLATE 132

Microphotographs of comparable section from the left kidneys of 3 rats (from previous studies) to illustrate differences in size and general structure. Each of the rats had a right nephrectomy prior to being fed the experimental diet.

FIG. 24. Rat on Liver Diet XIV for 156 days. Died uremic. Kidney weighed 8.52 gm. Microscopic examination showed: Extensive glomerular involvement and fibrosis; great cystic dilatation of tubules; large numbers of tubular casts (the very dark areas in the illustration). Classed as 4 plus nephritis in previous report. × 8.

FIG. 25. Rat on Liver Diet XIV for 208 days. Died uremic. Kidney weighed 5.40 gm. Microscopic examination: Similar to Figure 24 except that there was less cystic dilatation of the tubules and there were very few tubular casts. The difference in weight of the two kidneys is apparently due to a greater retention of urinary secretion in one than the other. Classed as 4 plus nephritis in previous report. × 8.

FIG. 26. Rat on Stock Diet II for 658 days. Animal in good condition when killed. Kidney weighed 2.17 gm. Microscopic examination: The organ presented a normal histological structure and was so reported in a previous article.



Medlar and Blatherwick Dietary Nephritis in the Rat

THE INFLUENCE OF ALLERGY ON THE DEVELOPMENT OF EARLY TUBERCULOUS LESIONS *

L. Dienes, M.D., and Tracy B. Mallory, M.D.

(From the Department of Pathology and Bacteriology, Massachusetts General Hospital, Boston, Mass.)

The influence of bacterial allergy in the form of tuberculin hypersensitiveness in accelerating the development of tubercle formation has long been generally recognized, and certain of its effects such as the intensification of inflammatory responses and the production of necrosis have been repeatedly emphasized. That allergy might play an even more fundamental rôle in the pathogenesis of the disease by determining, in part at least, the character of the anatomical lesions of tuberculosis has received little consideration. Yet, as the authors have pointed out in a previous paper,[1] the similarity of the histological reactions in most of the so-called infectious granulomas — syphilis, typhoid, glanders, brucellosis, tularemia and lymphogranuloma inguinale, for example — is so great that most pathologists candidly admit the dangers of confusion in routine diagnostic work. In all diseases of this group focal tissue reactions characterized by a predominance of large mononuclear phagocytes are the rule and in this same group of diseases bacterial allergy — defined as the first stage of the immune response to parenteral antigen, characteristic only of active as contrasted with passive immunity — is most regularly and persistently present and most highly developed.

Such a constant association of a morphological picture with an immunological state inevitably suggests the possibility of a causal relationship, though a more complicated interdependency cannot be excluded. If the relationship is a simple one, our alternatives are either that the presence of granulomatous lesions predispose to the development of a particularly high degree of bacterial allergy, or that bacterial allergy determines a focal mononuclear type of cytological response on the part of the host. That the latter actually occurs was the conclusion drawn by the authors in the work already cited and since confirmed in all essential details by Laporte.[2] Rössle,[3] too, was evidently thinking along closely similar lines when he recently stated that granulomatous reactions can

* Received for publication June 8, 1937.

occur only in diseases in which allergy develops. For a more complete discussion of the relationship of allergy, not only to cytological responses but to the production of antibodies, the reader is referred to the review recently published by one of the authors.[4]

So far as any theory of the histogenesis of the tubercle may be said to have received general acceptance, the tubercle is usually looked upon as analogous to a foreign body reaction to a parasite which is relatively indestructible and possesses a high lipoid content. Yet quantitative considerations of the amount of lipoid in a few hundred tubercle bacilli, compared to the amount of tubercular or other lipoid required to produce a noticeable foreign body reaction, obviously render such a hypothesis inadequate. Moreover, such an explanation could never even be promulgated to explain the nearly identical reactions to such different parasitic antigens as Gram-negative bacilli, spirochetes and filterable viruses.

The present work has, therefore, been undertaken to test further the applicability to tuberculosis of the hypothesis that allergy is the primary factor in determining the character of the early histological response to the infection in susceptible animals. If such a theory is tenable, it must be demonstrable (1) that the granulomatous lesions are not essential to the development of the delayed type of allergy, and (2) that tuberculin sensitivity develops at least as early as the appearance of the characteristic histological picture in the initial tuberculous lesion. The first of these points was demonstrated by Dienes [5] some years ago in the following manner. Guinea pigs were injected intratesticularly with a large dose of tubercle bacilli. Twenty-four hours later, when control histological examinations showed simple abscess formation with a practically pure polymorphonuclear response and without a trace of the granulomatous reaction considered characteristic of spontaneous or experimental tuberculosis, a simple protein such as egg albumin was injected into the testicle. After a second 24 hour period the testicle, still free from any trace of granulomatous reaction, was excised. The animals, however, proceeded to develop after 3 to 4 days a high grade of delayed hypersensitivity of the tuberculin type to the injected egg albumin. Furthermore, in a later work Dienes and Mallory [1] showed that typical, albeit rather feeble, delayed sensitivity to non-bacterial proteins could be regularly produced in uninfected animals. The present experiments are, therefore, devoted to testing the second of the imposed conditions

by an analysis of the time factors governing the development of hypersensitiveness and the appearance of mononuclear infiltration, fibroblastic proliferation and other factors of the granulomatous tissue response.

Experimental

Methods

The general plan of the experiments was as follows: Guinea pigs were heavily infected with large doses of tubercle bacilli to ensure rapid development of detectable tuberculin sensitivity. Pairs of animals were later reinfected with small doses of bacilli at suitably chosen times so that there were available for histological study reinfectious lesions produced from 1 to 8 days after the first infection and from 1 to 3 days old. The sites of inoculation and of reinfection were varied in different sets of experiments to control as far as possible the effect of varying tissue substrates, and lesions of the omentum, the skin, the loose tissues of the groin and of the testicle were studied. In a preliminary series of 10 animals the first infection was in the peritoneal cavity; in the 3 subsequent series, comprising in all 29 animals, the first infection was testicular. In the first 3 series of experiments the R_1 low-virulent strain of tubercle bacilli was used in 10 mg. doses for the primary infection, and in 0.1 to 2 gm. doses for the reinfectious lesions. In the final set of 9 animals 5 mg. of a freshly isolated, virulent human strain was employed for infection, and 0.1 mg. of the same strain for reinfection. All animals were skin tested with 0.01 cc. of an unheated synthetic medium tuberculin which gave no reaction in uninfected pigs to determine the first appearance of tuberculin sensitivity. At appropriate intervals the animals were killed, the lesions — primary infections, reinfections and tuberculin reactions — excised, fixed in Zenker's fluid, stained with hematoxylin and eosin or eosin-methylene blue, and with Ziehl-Neelsen's stain. Serial sections were not made but 3 sections at different levels from each block usually sufficed to give satisfactory pictures of the lesions. Since the relative distribution of granulocytes and mononuclear phagocytes characteristically showed a reciprocal relationship in proportion to the distance from the center of the lesion, care was exercised to use for study only sections that could be shown to pass close to the center of the lesion.

Experiments

In the preliminary series 20 mg. of the R_1 strain was injected intraperitoneally and at the same time a dose of 2 mg. was given intracutaneously. Pigs were killed on the 2nd, 3rd, 4th, 6th, 7th and 9th days. Each pig had been injected intracutaneously with a small dose varying from 2 to 0.1 mg. R_1 bacilli the day before death. The peritoneal, the original intracutaneous and the 24 hour cutaneous lesions were all examined grossly and histologically. From the gross point of view the peritoneal lesions developed, as was expected, primarily in the omentum, though in a few animals lesions were seen in the mesentery or the tunica as well. Up to and through the 3rd day the omentum, though rolled up into a ball, swollen and hyperemic, could be fairly easily untwisted by gentle traction, but by the 4th day isolated firm lesions became evident and on subsequent days dense fibrous adhesions bound it into a solid tumor-like mass. Microscopically by the 3rd day mononuclear cells predominated and from the 4th day onward fibroblastic proliferation was evident, which rapidly resulted in the development of abundant granulation tissue.

In each of the guinea pigs killed from the 3rd day onward two skin lesions were available for study, one made simultaneously with the intraperitoneal injection, the second 24 hours before the pig was sacrificed. In the 3 day pigs both the 72 and the 24 hours old reactions were similar in character though materially different in extent. The reaction in each consisted essentially of polymorphonuclear infiltration with very few mononuclears and no evidence of fibroblastic proliferation. Reactions from the 4th day on — the 24 hour as well as the older reaction — were entirely different in character. Polymorphonuclear leukocytes were still abundant and tended to be clustered fairly compactly about the bacilli, but the surrounding tissue showed an extensive reaction with many mononuclear phagocytes hovering about the edges of the lesions and clumped about the blood vessels and nerve sheaths.

This preliminary set of animals served to emphasize the significance of the period from the 3rd to the 5th day, and in the subsequent series attention was especially concentrated on this period.

In several of these animals an interesting phenomenon characterized by a fiery red, hemorrhagic reaction of the parietal peri-

toneum developed. A similar local hemorrhagic reaction was noted in some of the animals described below in the group recorded as Series III, which were infected in the loose tissues of the groin.

A brief description of this phenomenon seems justified since we are unaware of any published report of a similar phenomenon in uncomplicated tuberculous infections, though Dienes [6] has reported a similar lesion following the injection of egg white in tuberculous animals previously infected and sensitized by the intraperitoneal route. It was observed in about half of the intraperitoneally infected animals, which were sacrificed on the 5th, 6th and 7th days after infection, and in one instance was observed by laparotomy under local anesthesia to rule out agonal phenomena. The reaction consisted of multiple small hemorrhages, sometimes nearly confluent, in the parietal peritoneum, which ordinarily shows little or no gross change at this stage of intraperitoneal infection in contrast with the omentum which is heavily beset with lesions. It was particularly evident where bits of omentum or of exudate had become adherent to the peritoneum. Microscopically these areas showed in addition to the hemorrhage intense monocytic infiltration, but no polymorphonuclear accumulation such as invariably is associated with the presence of the organisms themselves (Fig. 12).

In the guinea pigs of Series III a 5 mg. primary dose was given simultaneously in the groin and in the testicle. In the latter no hemorrhagic phenomenon appeared, but in the former 3 days after infection a zone of hemorrhage 30 mm. in diameter developed, which reached its maximum on the 4th day and then slowly disappeared during the next 4 days. A similar reaction has been noted in rabbits infected in the subcutaneous tissues.

Both the peritoneal and the subcutaneous lesions resemble closely both grossly and microscopically the flaring up of infection sites at the moment when allergy develops, and also the similar reactions recently reported from this laboratory following injection of human serum and of turtle egg in guinea pigs.[7]

It seems evident that these reactions are allergic in character and it is suggested that they indicate diffusion for some distance from the lesions through the tissues of tuberculin-active substances. This in turn renders possible the early sensitization of tissues in the immediate neighborhood of a lesion before generalized sensitivity develops.

Such features of the subsequent 3 series of experiments as are susceptible of tabulation are presented in Table I. From this it will be evident that no animal proved tuberculin-sensitive on the 2nd day, and no animal failed to show definite sensitivity on the 4th day. The reactions on the 3rd day appeared negative or equivocal on gross examination. When examined microscopically, using the criterion of significant mononuclear infiltration as evidence of a positive reaction, 3 out of the 7 examined microscopically were considered weakly positive.

The histological preparations of the tuberculin reactions showed the picture that we have previously described [1]; a very slight and presumably non-specific reaction to the injected fluid characterized by a small number of scattered polymorphonuclear leukocytes in the 1st and 2nd day, and most of the 3rd day animals; marked constant mononuclear infiltration in all animals tested from the 4th day onward, with some tendency to the reappearance of polymorphonuclears as the degree of sensitiveness became more intense and necrosis appeared in the tissues. In a recent paper which essentially confirms our observations Laporte [2] describes edema and polymorphonuclear infiltration as the first stage of the histological process in a tuberculin reaction. At a later point in the paper he describes a similar result from injecting tuberculin into normal animals. Our findings are entirely in accord with his but we prefer to regard this reaction as a non-specific response to the trauma of the injection and to the faintly irritating effect of all tuberculin preparations rather than as an integral part of the tuberculin reaction.

In the reinfectious lesions (*i.e.*, intracutaneous or intratesticular injections of living tubercle bacilli in a previously infected pig) the invariable initial response was a flood of polymorphonuclear leukocytes which quickly clustered about the organisms with the formation of small abscesses. When the reinfection was produced 2 days after the primary lesion nothing more was noted, but in some of the 3 day examples, and constantly from the 4th day on, a noticeable alteration in the reaction appeared. There was greater hyperemia, the fibroblasts of the surrounding tissues appeared swollen and their nuclei hyperchromatic; in the early stages an occasional mitotic figure, and in the later stages numerous mitoses were found. Simultaneously, as far as we could judge, large num-

TABLE I

Day	Series I: Primary infection — 10 mg. R_1 intratesticular; Reinfection — 0.1 mg. R_1 intracutaneous								Series II: Primary infection — 10 mg. R_1 intratesticular; Reinfections — 0.1 mg. intracutaneous								Series III: Primary infection — 5 mg. virulent Tb., testicle or groin; Reinfections — 0.05 mg. virulent Tb., skin and testicle							
	Primary lesion		Tuberculin			R_1 intracutaneous			Primary lesion		Tuberculin			R_1 intracutaneous			Primary lesion		Tuberculin			Virulent Tb. intracutaneous		
	P	M	Gross	P	M	Gross	P	M	P	M	Gross	P	M	Gross	P	M	P	M	Gross	P	M	Gross	P	M
2	+++	±	3 x 4	±	±	3 x 4	+	o	+++	±	Trace	±	o	o	++	±	+++	o	o			o	++	±
	++	±	5 x 6	±	±	5 x 6	+	o	+++	±	Trace	±	o	o	++	±	+++	±	o			Trace	++	o
3	++	++	3 x 4	±	+	5 x 6	++	+	+++	++	Trace	±	+	o	++	+	+++	++	o			Trace		
	++	++	5 x 6	±	+	5 x 6	++	+	+++	++	Trace	±	±	o	++	+			o			Trace	++	o
											Trace	±	±	Trace	++	±								
4									+++	+++	7 x 7	±	+	10 x 8	++	++	+++	+++	8 x 7			8 x 7	+	+
									+++	+++	7 x 6	±	++	9 x 8	++	++	+++	+++	8 x 8			8 x 8	++	++
5	++	+++	14 x 13	+	+++	12 x 10	++	+++	+++	+++	13 x 13	±	+++	14 x 13	+++	++	+++	+++	15 x 13			11 x 10		
	++	+++	11 x 10	+	+++	10 x 10	++	+++	+++	+++	8 x 9	±	+++	13 x 11	+++	++	+++	+++	15 x 13			12 x 10	++	++
6	++	+++	13 x 12	+	+++	10 x 8	++	+++																
	++	+++	13 x 11	+	+++	12 x 11	++	+++																
8	++	+++	20 x 20	+	+++	15 x 10	++	+++																
	++	+++	20 x 18	+	+++	16 x 15	++	+++																

The letters P and M heading the columns stand respectively for polymorphonuclear leukocytes and large mononuclear phagocytes.
The proportions of these types of infiltrating cells have been roughly estimated by four grades from ± to +++.
The figures in the columns labelled "Gross" indicate the size of the discolored area in the skin in millimeters.

bers of mononuclear phagocytes appeared, first in the surrounding tissues at some distance from the organisms and polymorphonuclear leukocytes, but fairly quickly condensing in a layer several cells thick just outside the neutrophils to form a loosely encapsulating wall. This process first became manifest on the 3rd day in a few only of the animals. By the 4th day, when hypersensitivity is well marked, it was never lacking. Figure 1 illustrates the localized abscess which regularly develops when living tubercle bacilli in doses from 0.1 to 2 mg. are injected intracutaneously into either sensitized or non-sensitized animals. This particular one was produced in a non-sensitized animal with a dose of 2 mg. and was excised on the 3rd day. Sensitization as judged by the tuberculin test had not yet developed. Figure 2 is a higher power of the corium surrounding the abscess and shows that even on the 3rd day the cellular reaction in this instance was almost purely polymorphonuclear. In contrast, Figures 3 and 4, likewise from the corium just outside the central abscess, but from a 24 hour old reaction in a pig sensitized 4 days before the intracutaneous reinfection, show very few polymorphonuclears, marked monocytic infiltration and definite proliferation of fibrous tissue. Reinfectious lesions in the testicle developed in an essentially similar manner to those of the skin. Such lesions were produced in the guinea pigs recorded as Series II in the tabulated summary by the injection of 0.2 mg. of R_1 bacilli in the opposite testicle from that used for the primary infection. Figure 5 shows the exudate at 24 hours in a testicle that was infected 2 days after the first infection. The exudate consists almost wholly of infiltrated polymorphonuclears and mononuclear cells are entirely lacking. Figure 6, made again from a 24 hour lesion in a testicle but one where reinfection occurred 4 days after the first infection, shows large areas of tissue densely infiltrated with mononuclear leukocytes.

A comparison of reinfectious lesions with primary infections, when interpreted in the light of our previous studies of the mononuclear character of the cellular response to tuberculin hypersensitivity, clearly showed a change in the character of the reaction on the 3rd day. At this time the reaction to tuberculin was not usually clearly positive. Probably the non-resorbability of the bacilli themselves makes them a more sensitive reagent for determining the first trace of hypersensitivity than tuberculin.

The Primary Infection

The development of the lesions at the sites of the massive primary infections in the testicles, and for that matter in the soft tissues of the groin in Series II, proved remarkably uniform. During the relatively short periods over which the animals were observed, no noticeable difference was seen between the lesions produced by the R_1 and the virulent strain. After 24 hours the bacilli were found enclosed in an abundant exudate consisting entirely of polymorphonuclear leukocytes (Fig. 7), while the surrounding tissue showed marked hyperemia and edema. At 48 hours distinct changes could be seen in the fibroblasts; they appeared much larger and more prominent, the nuclei stained more intensely, and the cytoplasm had increased in volume. By 72 hours distinct progression of the lesion had occurred (Figs. 8 and 11). Mononuclear phagocytes were appearing in abundance, the connective tissue cells had clearly multiplied, and occasional mitotic figures were encountered. By the 4th day a thick wall of mononuclears and young fibroblasts surrounded and encapsulated the abscesses. Mononuclears began to penetrate the abscess and to replace the polymorphonuclears. By the 5th day the granulation tissue had extended far from the site of injection and formed wide bands between the atrophying parenchymal tubules (Fig. 9). The lesions continued to advance until the 8th day, the last one studied, with progressive replacement of the abscesses with tuberculous granulation tissue. Only on the last 2 days could the development of "epithelioid cells" from the mononuclears be made out.

Minor variations in this pattern were observed. In Series II, for instance, there was a distinctly greater connective tissue reaction on the 2nd day than in any of the other sets of animals. In the group where one of the primary sites of infection was the groin, an area of hemorrhage 30 mm. in diameter appeared on the 3rd day. This did not, however, appear to modify the usual progress toward encapsulation of the lesion.

Summary of Experimental Evidence

The experimental evidence gathered from study of the tuberculin reactions and the reinfections may be summarized as follows: At a point not later than the 4th day, sometimes even on the 3rd

day, the reaction of the animals to tuberculin — usually more evidently to tubercle bacilli themselves — is clearly different in character from that observed in uninfected animals or in the first 2 days after the primary infection. It is quicker, far more intense in proportion to the dosage used, and microscopically can be shown to have the predominantly mononuclear character previously described [1] as characteristic of the delayed type of hypersensitivity. Even at this early stage the animals are clearly developing generalized tuberculin sensitivity.

At approximately the same time period — regularly on the 4th day, usually in incipient form on the 3rd day — a distinct change in the character of the primary lesion can be demonstrated. Whereas the reaction of the first 24 to 48 hours shows only congestion and serous exudation with marked accumulation of polymorphonuclear leukocytes — a reaction indistinguishable from that which might be produced by any pyogenic organism — beginning sometimes on the 3rd day and becoming well marked on the 4th, a second type of reaction has become obvious. Collections of large mononuclear phagocytes begin to appear as cuffs about the blood vessels and nerves, to infiltrate the stroma first at some distance from organisms and leukocytes but gradually to condense about them to form a wall several cells thick. At about this time the connective tissue cells begin to appear swollen and hyperchromatic, and mitotic figures begin to appear in abundance in them. In another 24 hours collagen begins to be laid down and true encapsulation is initiated. The parallelism between the appearance of demonstrable hypersensitivity and the alteration of the character of primary lesion are closer when both inoculations are made into similar tissues. When one injection is made into the peritoneal cavity and the other into the skin, for instance, noticeable differences in the rate of development of the lesions are apparent. The complications introduced by the absorption of large amounts of fluid exudate might readily explain the discrepancies however.

Discussion

Under the conditions of the experiments that have been described it has been shown that a very close parallelism exists between the appearance of demonstrable tuberculin sensitiveness and the shift from a purulent to a granulomatous infiltration of the

tissues. More exact determination of the time factors seems scarcely feasible when one reflects that a faint skin reaction of the delayed type can only be read 24 hours after it is performed — an interval during which it is fair to assume that the degree of hypersensitivity has been progressively and rapidly increasing. The discrepancies observed, therefore, seem to fall within the limits of error of the method and it is not unreasonable to conclude that hypersensitivity and alteration in the character of the histological response — an alteration which for the first time begins to suggest the typical granulomatous lesion — appear simultaneously.

It is realized, of course, that the massive infections used in these experiments have no counterpart in spontaneous tuberculosis of man or beast, but purposeful exaggeration of natural phenomena is one of the most useful methods of studying them and has been used by choice or necessity in most of the experimental work on the disease. A negative result from our experiments would have been strong evidence against the validity of our thesis. The result obtained is of value as strongly suggestive confirmatory evidence, but does not of itself constitute proof of similar time relationships under conditions of minimal infection. With the usual "spontaneous" infection of man or animal it is certain that weeks, probable that months may pass before generalized tuberculin sensitivity develops.

Obviously, to maintain our thesis we must fall back on the theory of local hypersensitiveness, always a favorite theme of speculation but except in special instances tantalizingly difficult to demonstrate experimentally.

Certain observations of Stewart are of great interest in this regard. In a study [8] of the histology of tuberculous infections of the testicle in guinea pigs rendered allergic with preliminary doses of heat-killed tubercle bacilli he noticed that attempts at healing — judged primarily on the basis of fibroblastic proliferation — became evident several days before generalized hypersensitivity could be recognized by skin tests. A probable explanation for this seemed to him to be the local development of allergy before it became generally detectable. In a second investigation [9] he was able to demonstrate this by the injection of tuberculin directly into the infected testis. A reaction characterized by extensive necrosis of the tubular cells, and an acute inflammatory reaction with hem-

orrhage and fibrin deposit appeared regularly when the injection was made on the 3rd day and became more marked on subsequent days. It was not until the 11th day that he observed positive reactions from tuberculin injections into the other uninfected testicle or in the skin. The time relationships of slight reactions on the 3rd day, well marked ones from the 4th day onward, are remarkably close to those we have demonstrated by a different technique.

Other forms of localized hypersensitivity, less directly applicable to tuberculosis but significant because of the more perfect control which is possible, are well known. The cutaneous drug-idiosyncracies, for instance, are known frequently to remain limited to the segment of skin originally exposed. Even protein sensitizations may remain localized to the treated area, as in the experiments of Redfern [10] and of Simon and Rackemann.[11] Not only sensitization but immunization, as represented by the presence of antibodies, has been shown on occasion to develop locally or to remain localized. Smith, Orcutt and Little,[12] for instance, showed that after inoculation of *Br. abortus* in one quarter of a cow's udder the antibodies appeared in highest concentration in the infected quarter. Recently McMaster and Hudack [13] observed that after injection of bacteria into the ear of mice agglutinin appeared first in the corresponding cervical lymph node where it could be detected for some time in higher concentration than in the blood serum or in extracts of other lymph nodes. In the light of our theory that bacterial allergy represents the first phase of the normal immune process, it is only reasonable to suppose that it also should appear locally earlier and in higher degree than general sensitivity.

It would be unwise to conclude without pointing out certain limitations to what we feel are justifiable deductions from our experiments. We are not attempting to maintain and do not believe it tenable that allergy is the only cause of mononuclear infiltration or of granulomatous reactions. The response to agar-agar or to lipoid injections is too obvious evidence to the contrary. Nor would we wish to be understood as refusing to credit to the lipoids of the tubercle bacillus or to those developing in the course of caseation a rôle in the determination of the later stages of the cytology of the tubercle. The rôle of allergy in the early stages of tuberculosis lies in the greatly increased rapidity and dispropor-

tionate intensity with which the granulomatous response develops, a rôle that is certainly not without importance for the understanding of the disease.

Summary

A group of experiments designed to study the correlation of the early histological response to tuberculous infection with the development of allergy has been described and illustrated. In guinea pigs with massive primary infections of the peritoneal cavity or testicles, reinfectious lesions were produced on successive days and the animals simultaneously skin tested for tuberculin sensitivity. After the lapse of varying periods of time the primary and reinfectious lesions and the tuberculin reactions were compared histologically. Under the conditions employed and within the limits of error of the method, it was found that the first appearance of significant numbers of large mononuclear cells and the development of detectable tuberculin sensitivity occurred simultaneously between the 3rd and 4th days after the primary infection. The significance of these observations in relation to the authors' previously advanced hypothesis that bacterial allergy is responsible for the early granulomatous reaction of the host has been discussed.

REFERENCES

1. Dienes, Louis, and Mallory, Tracy B. Histological studies of hypersensitive reactions. *Am. J. Path.*, 1932, **8**, 689–708.

2. Laporte, R. Histo-cytologie des réactions locales d'hypersensibilité chez le cobaye (réactions allergiques à la tuberculine et réactions anaphylactiques). *Ann. Inst. Pasteur*, 1934, **53**, 598–640.

3. Rössle, R. Die nosologische Stellung des Rheumatismus. *Klin. Wchnschr.*, 1936, **15**, 809–814.

4. Dienes, L. The specific immunity response and the healing of infectious diseases. Significance of active immunity and the connections between the immunity response and the anatomic lesions. *Arch. Path.*, 1936, **21**, 357–386.

5. Dienes, L. Ueber die Wirkung des tuberkulösen Krankheitsherdes auf die Immunitätsreaktionen des Organismus. *Ztschr. f. Immunitätsforsch. u. exper. Therap.*, 1930, **68**, 13–42.

6. Dienes, L. Hemorrhagic reactions in tuberculous lesions and skin tests during protracted anaphylactic shock. *Proc. Soc. Exper. Biol. & Med.*, 1930, **27**, 690–692.

7. Dienes, L., and Simon, F. A. The flaring up of injection sites in allergic guinea pigs. *J. Immunol.*, 1935, **28**, 321–330.

8. Stewart, Fred W. Testicle infection in guinea pigs sensitized with killed tubercle bacilli. *J. Immunol.*, 1926, **11**, 123–134.

9. Stewart, Fred W. Observations on focal tuberculin hypersensitiveness in an infected organ previous to a general sensitization. *Am. J. Path.*, 1925, **1**, 495–501.

10. Redfern, William W. Skin reactions produced by antihuman serum. *J. Immunol.*, 1930, **18**, 109–120.

11. Simon, Frank A., and Rackemann, Francis M. The development of hypersensitiveness in man. I. Following intradermal injection of the antigen. *J. Allergy*, 1934, **5**, 439–450.

12. Smith, Theobald, Orcutt, Marion L., and Little, Ralph B. The source of agglutinins in the milk of cows. *J. Exper. Med.*, 1923, **37**, 153–174.

13. McMaster, Philip D., and Hudack, Stephen S. The formation of agglutinins within lymph nodes. *J. Exper. Med.*, 1935, **61**, 783–805.

DESCRIPTION OF PLATES

PLATE 133

FIG. 1. The abscess produced by the intracutaneous injection of 2 mg. of R_1 tubercle bacilli in a previously normal guinea pig. It was excised on the 3rd day after infection. × 20.

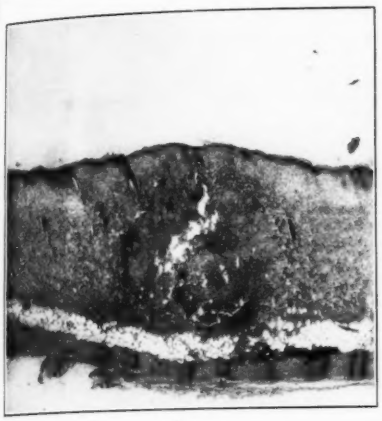
FIG. 2. A field from the corium at a little distance from the central abscess in this same case. The inflammatory response still consists entirely of polymorphonuclear leukocytes. × 100.

FIG. 3. A field from the corium bordering the central abscess produced by the intracutaneous injection of 0.1 mg. of R_1 tubercle bacilli. The guinea pig had been primarily infected 4 days earlier with 10 mg. of R_1 bacilli in the testicle, and the skin lesion was excised at the end of 24 hours. It shows rare polymorphonuclears, numerous monocytes and an increase in fibroblasts. × 100.

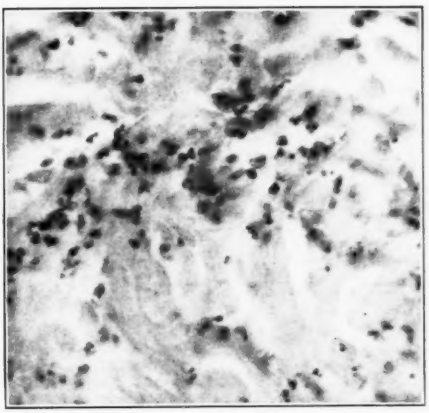
FIG. 4. The same. × 300.

FIG. 5. Exudate in the testicle some distance from the central abscess 24 hours after the injection of 0.1 mg. R_1 bacilli in a guinea pig primarily infected 2 days before with 10 mg. of tubercle bacilli in the opposite testicle. A simultaneous tuberculin test was negative. The great predominance of polymorphonuclears and the paucity of mononuclears are evident. × 250.

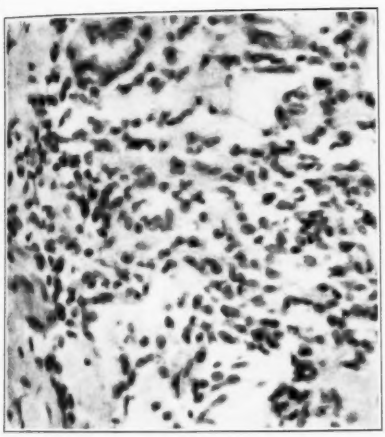
FIG. 6. A corresponding field from a 24 hour old testicular lesion of a slightly allergic animal in which the reinfectious lesion was produced 4 days after the primary infection. The reaction at this spot is almost wholly mononuclear. × 250.



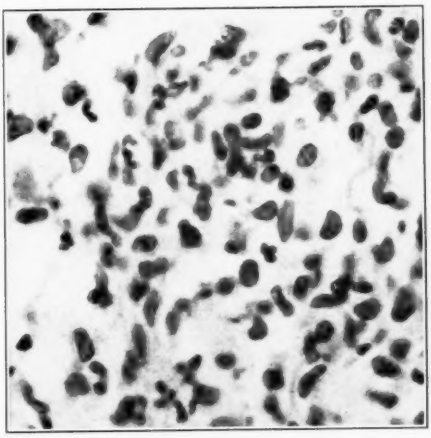
1



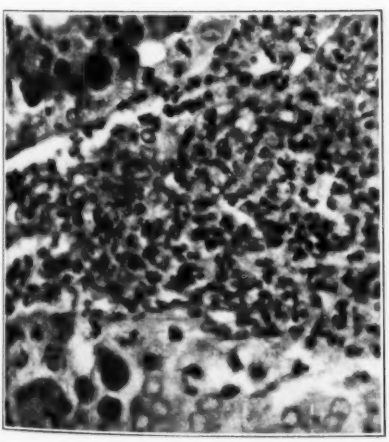
2



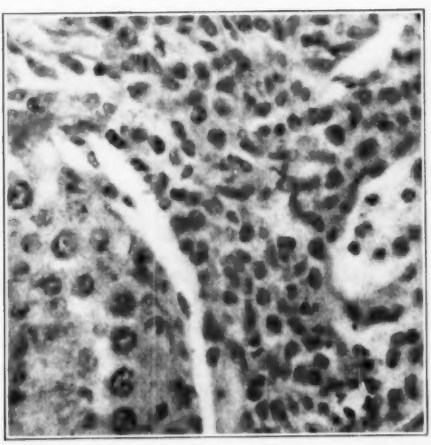
3



4



5



6

PLATE 134

FIGS. 7, 8, 9 and 10 show primary lesions of the testicle produced by infection with 5 mg. of R_1 tubercle bacilli. They are respectively 1, 3, 5 and 8 days old.

Fig. 7. At 1 day a poorly defined abscess only is apparent.

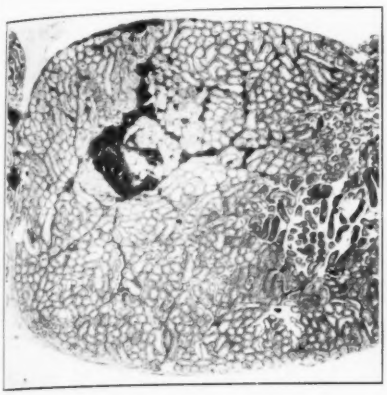
Fig. 8. At 3 days a narrow rim of mononuclear infiltration and granulation tissue is evident.

Fig. 9. At 5 days the granulomatous encapsulation is broad and extends in narrow bands out into the surrounding parenchyma.

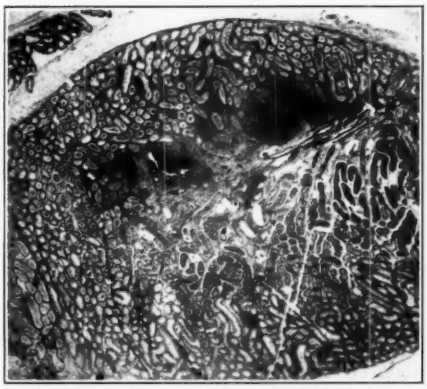
Fig. 10. At 8 days the abscess has been completely replaced by the granulomatous mass which occupies nearly the entire testicle. × 6.

FIG. 11. A portion of the reacting tissue outside the abscess from the same testicle as Fig. 8, a 3 day lesion. In this area the reaction consists almost solely of mononuclear phagocytes. × 250.

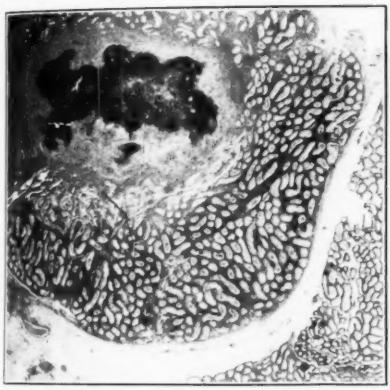
FIG. 12. The peritoneum of an intraperitoneally infected pig which shows the hemorrhagic reactions described in the test. In this area marked mononuclear infiltration is present. × 100.



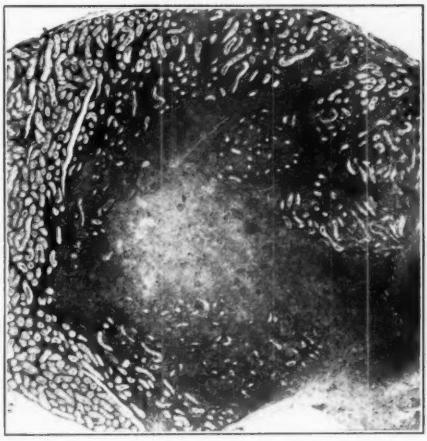
7



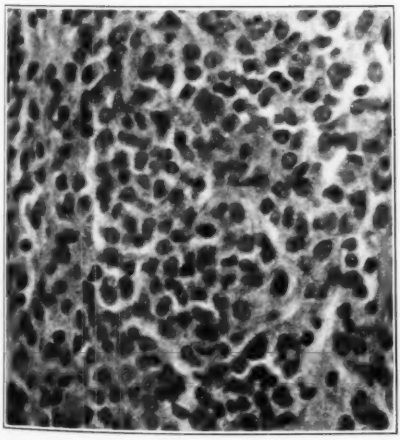
8



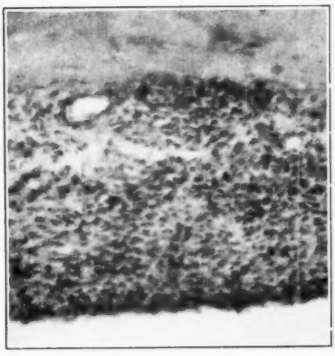
9



10



11



12

ENCEPHALITIS AND MENINGITIS IN THE CHICK EMBRYO FOLLOWING INOCULATION OF THE CHORIO-ALLANTOIC MEMBRANE WITH H. INFLUENZAE *

MAE GALLAVAN, M.D.

(From the Department of Pathology, Vanderbilt University Medical School, Nashville, Tenn.)

The use of the embryo chick for the study of bacterial infection was suggested by Goodpasture [1] in 1933. The report of Goodpasture and Anderson [2] on bacterial invasion of the chorio-allantoic membrane indicated that this method could be applied to the study of several types of microorganisms. The investigation of Buddingh and Polk [3] on meningococcus infection of the chick embryo further demonstrated its value for the study of the pathogenesis of this infection and suggested the use of this method for the analysis of the immunity factors in infection.

This report concerns an investigation of *Haemophilus influenzae* infection, using in general the methods and approach indicated by these workers. The use of the chick embryo has been extended to a comparative study of the pathogenesis of infection with various strains of *H. influenzae*, and of the effect of successive transfers through this host on the microorganisms and the infection which they produce.

EXPERIMENTAL

Sources of Organisms

Eight strains of *H. influenzae* were investigated.† Six had been isolated from autopsy material within the 48 hours previous to

* Aided by grants from the Josiah Macy, Jr. Foundation and the Division of Medical Sciences, Rockefeller Foundation.

† The organisms were obtained from the following sources: The number refers to the autopsy, 1937, Vanderbilt Hospital. *14* = 48 hour ascitic broth culture of spinal fluid from a patient who later died of meningitis; *15* = pleural exudate obtained at autopsy 1 hour after death from *H. influenzae* pneumonia; *16* = 24 hour blood agar culture of exudate from a lung showing also *Str. viridans* and a hemolytic staphylococcus obtained at autopsy 3 hours postmortem; *32P* = 24 hour blood agar culture of pleural exudate from an autopsy 5 hours after death from pneumonia and meningitis; *32M* and *32MC* = meningeal exudate from the same autopsy; *37* = meningeal exudate obtained at autopsy 5 hours postmortem; *55* = 24 hour blood agar culture of meningeal exudate from an autopsy 6 hours after death; and *P* = strain furnished by Dr. Caroline Chandler, isolated from a spinal fluid in 1934 and cultured in defibrinated rabbits' blood.

Received for publication July 10, 1937.

their inoculation onto the egg. Of these, Nos. *15* and *32P* were from pleural exudate, *16* from a pneumonic lung, and *32* (*M* and *MC*), *37* and *55* were from meningeal exudates. Two strains were from the spinal fluids of patients; *14* isolated 72 hours previous to inoculation, and *P* isolated in 1934. The latter strain was supplied by Dr. Caroline Chandler.

Characteristics of the Organisms Before Inoculation

All strains were Gram-negative bacilli, which reduced nitrates, fermented dextrose, and required both the X and V factors for growth, as determined by their failure to grow in either autoclaved Levinthal broth or yeast extract broth. In determining the cultural, biochemical and immunological characteristics, and the pathogenicity of each strain, the reports of Pittman [4] and of Fothergill and Chandler [5] were largely followed.

With the exception of *16*, all strains produced on Levinthal agar plates the giant iridescent colony typical of the S type. Strain *16* produced a giant colony similar to the S type in all respects except that it was never distinctly iridescent. Strains *32P* and *32MC* fermented galactose slightly. Strains *14*, *15*, *16*, *32M* and *P* fermented xylose slightly. No gas was produced in any of the eight sugars used.[4] Indol was produced by all with the exception of Strain *16*.

Agglutination tests performed by the ordinary method with polyvalent horse serum gave unquestionable agglutinations only in serum dilutions 1:20, and occasionally in dilutions of 1:40 and 1:80, against all strains except *32MC*. The latter gave definite agglutinations in serum dilutions through 1:1280. Clearer results were obtained by the thread reaction [5]; there was a sharp endpoint with all strains in serum dilutions varying between 1:320 and 1:1280. A positive precipitative reaction was obtained against polyvalent horse serum through 1:4 dilutions of the filtrate from the cultures of Strains *14*, *32P* and *37*; through a 1:8 dilution of filtrate from *15*, *32MC* and *32M*; and a 1:32 dilution of *P* filtrate. No definite precipitative reaction was obtained with Strain *16* filtrate.

The pathogenicity of each of these strains for mice and the reactions produced by the intradermal injection of rabbits were comparable with those reported for S strains of the organisms.[4] Death of mice was produced within 24 hours following the intra-

peritoneal injection of 0.5 cc. of a 20 hour culture grown in an Erlenmeyer flask of Levinthal broth (2.5 per cent blood). One tenth cc. of this culture introduced superficially into a rabbit's skin produced within 24 hours an elevated, definitely erythematous area about 2 cm. in diameter, which became indurated and remained from 1 to 2 weeks.

Experimental Procedure

The chorio-allantoic membranes of chick embryos were exposed according to the coverslip method described by Goodpasture and Buddingh.[6] Series *14, 15* and *16* were each started by inoculating the membranes of 6 11 day eggs with a platinum loopful of a 24 hour Levinthal agar culture of the organisms. Similarly, for Series *32M* meningeal exudate was directly inoculated, and for series *P* a 24 hour culture in defibrinated rabbit's blood was used. After inoculation the eggs were returned to the incubator.

Twenty-four hours later smears were made of exudates removed from the membranes with a platinum loop. These smears were stained by Wright's or Gram's method. A succeeding generation of a strain was started by transferring exudate from that membrane of the preceding generation which showed by smear the most profuse growth. A culture on a Levinthal agar plate was also made from this exudate. If available, 6 11 day eggs were used for each generation; sometimes fewer or older embryos had to be used. Occasionally, on account of fluctuations in the supply of fertile eggs, the interval between generations was greater than 24 hours; the greatest interval was 6 days.

In order to study the pathological changes produced by these strains originally, embryos were sacrificed at different stages of infection. An attempt was made to obtain a representative for each 24 hours postinoculation of each strain. As the survival period of the embryos of the different generations and series varied, and since the number in each generation was relatively small, several generations had to be drawn upon in order to obtain a complete group. The number in each group was limited by the duration of survival after infection, which varied from 4 to 10 days. These embryos and their chorio-allantoic membranes were fixed in Zenker's fluid with 10 per cent acetic acid. After removing the legs and wings, transverse sections of the entire fixed embryos were cut

and sections were stained by the hematoxylin-eosin and Giemsa methods.

With the strains later obtained the embryos for microscopic study were all taken from the same generation, for which 12 to 25 11 day embryos were inoculated. This was done at the 5th generation of *32M*. Subsequently an attempt was made to give the same infecting dose to each member of the large generation to be used for sections. In Series *32MC* and *32P* the organisms from a 24 hour Levinthal agar subculture were suspended in sterile chick amniotic fluid. A drop of this suspension was dropped from a capillary pipette onto each membrane. Likewise, for the embryos for section, the third generation of *16B* and the second of *55* were inoculated with a mixture of the membranal exudate and allantoic fluid of the egg chosen from the preceding generation. In Series *37* the embryos for study were inoculated with a loopful of the meningeal exudate.

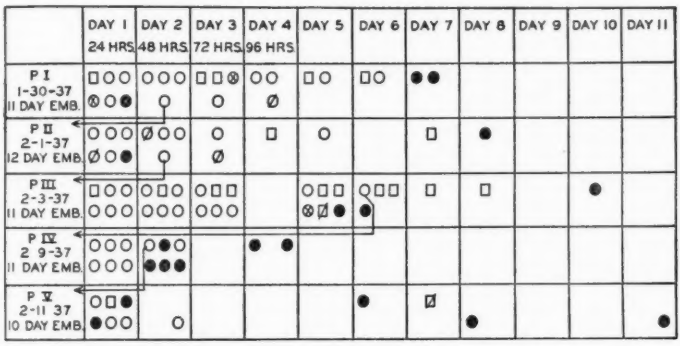
Routine smears of the exudate from the membrane, and cultures on Levinthal agar from the membrane and heart's blood, were made at the time of sacrifice of each embryo of these larger generations.

Strains *37* and *55* were not transmitted through further generations. All other strains were transferred through 20 generations of chorio-allantoic membranes. The only contamination in the transfer series was of Strain *14* with staphylococcus in the 12th generation. This mixed growth was transferred through the 13th and 14th generations; the 15th generation was inoculated from a typical S colony of a 16 hour culture of the exudate of Generation 14 on Levinthal agar. With the exception of Strain *16*, a sufficient number of the embryos survived the infection long enough to make the maintenance of the strains through successive generations and the investigation into the pathological process easily possible. During one of the periods when no fertile eggs over 10 days old were available, all the embryos of Series *16* died; the strain from a culture of the last (9th) generation was maintained for 4 days by daily transfers on Levinthal media. A new series (*16B*) was then started by inoculating 6 11 day embryos from this 4th Levinthal agar culture of the 9th egg generation of *16*.

At the 20th generation another series of embryos was taken from each strain for microscopic study of the infection at 24 hour

intervals after inoculation. For this purpose from 12 to 25 11 day chorio-allantoic membranes were inoculated with a drop of the suspension of the membranal exudate in the allantoic fluid from the chosen 19th generation egg. To confirm initial infection Levinthal agar plate cultures were made from the exudate of each 20th generation membrane 24 hours after inoculation; all negative eggs were discarded. The sacrificed embryos were treated as described for the earlier generation.

A record of each series was kept according to the following chart, which is an elaboration of that used by Buddingh and Polk.



EMB - EMBRYOS

○ EMBRYO SHOWING BACTERIA ON THE MEMBRANE.

□ EMBRYO SHOWING NO BACTERIA ON THE MEMBRANE.

⊗ EMBRYO SHOWING CONTAMINATION OF THE MEMBRANE.

Ø Ø SACRIFICED EMBRYOS.

● DEAD EMBRYO.

CHART 1. Each horizontal row, divided into 24 hour intervals, represents 1 generation of inoculated embryos. The strain, generation number, date of inoculation and age of the embryos used, are noted in the first square. The arrow indicates the embryo from which the succeeding generation was inoculated.

The culture, biochemical and immunological characteristics and the pathogenicity were determined for each of the 19th generation strains used to inoculate the 20th generation.

Cultures of each original strain were carried through defibrinated rabbit's blood and the incubation and time of transfer were the same as for the egg series. These cultures were studied as controls.

Effect on the Characteristics of H. influenzae of Transference Through 20 Generations on the Chorio-Allantoic Membrane of the Chick Embryo

There was no change in the toxicity of the strains to mice or in the skin reaction of rabbits. There was no alteration of the agglutination by polyvalent serum with Strains *16B, 32M, 32MC* or *P*. Strains *32P* and *15* originally gave an ordinary agglutination reaction in serum dilution through 1:40; after the 20th egg generation these strains gave definite agglutinations through serum dilutions of 1:5120; the control 20th generation rabbits' blood cultures did not show an increase in agglutinability. The end-point of the thread reaction was in the next serum dilution above that shown originally for each strain. The precipitative reaction end-point was consistently in the next lower dilution of the filtrate than it was originally. Morphologically the organisms were indistinguishable. Except for a marked diminution in iridescence in the 8th and 9th generations of Strain *14*, the colonies on Levinthal agar plates throughout the 20 generations of all strains showed the original characteristics.

Studies of the Inoculated Chorio-Allantoic Membrane

The gross lesions on the membrane varied greatly, even within the same generation, and no characteristic appearance for any one strain was noted. Usually within 24 hours after inoculation the membrane became edematous and moist. The process progressed during the following 24 to 48 hours (Fig. 1), then regressed, leaving finally a dry, slightly thickened membrane at the end of 6 days. One or more areas of pale yellow, moderately thick exudate were found; during the edematous stage these areas frequently appeared as slight depressions and remained as small crusted areas on the dry membrane. Frequently, although at the time of mechanically exposing the membrane no trauma was evident, 24 hours after inoculation the outline of the square window could be traced as lines of purulent exudate on the membrane. Occasionally an increased purulent exudation produced a heaped up mass which became a crusted nodule as the membrane dried.

Smears made of the material obtained by scraping the membrane with a platinum loop and stained with Wright's stain showed an

inconstant picture. The exudate usually consisted of red blood cells, mononuclear cells, polymorphonuclear leukocytes and bacteria. The relative numbers of these varied in the exudate of the same series, even in the same generations, and there was nothing by which the strain could be differentiated. With very few exceptions mononuclear cells predominated. Lymphocytes were rarely found; the majority of the mononuclear cells were monocytes with the characteristic purple loose nucleus and pale blue cytoplasm, which in the early stages of infection contained large eosinophilic masses. These masses were also found free and probably were hyaline bodies from ruptured degenerating epithelial cells. Another quite similar type of granulated cell, which by some methods showed a poorly stained nucleus, suggested desquamated degenerating epithelial cells. There were a few deeply staining, compact mononuclear cells of variable size, sometimes with pale blue nucleoli and usually containing a few, large, purplish red granules which were classified as myelocytes. The polymorphonuclear cells contained large fusiform eosinophilic granules.

All strains of bacteria showed some pleomorphism with a tendency to greater variation in morphology as the infection persisted. The degree of degeneration of the bacteria varied inconstantly; sometimes even within 24 hours most of the bacteria appeared as oval masses which stained only peripherally (Fig. 2); in other instances even at 96 hours the organisms were well preserved, regular and deeply staining. All strains showed intracellular organisms in most of the smears; this was less notable, however, with the stock strain *P*. In the early stages of infection (24 to 48 hours) cells were found distended with a mass of deeply staining bacilli, the nucleus being flattened against the periphery (Fig. 3). Such cells could not always be identified but the majority were monocytes. Other monocytes contained fewer bacteria which sometimes were thick and pale staining; occasionally a small deeply staining mass of bacteria was separated from the mass of blue cytoplasm by a clear zone. In the latter and in disrupted cells of the first described type tiny eosinophilic rods were found. As the infection persisted fewer monocytes containing bacteria were found. Of these, the relative number containing a few, large bacillary forms increased; in the others the intracellular masses of bacteria became predominantly eosinophilic. Usually by the

5th day of infection only an occasional cell containing a few bacilli was found. In those instances in which the bacteria showed degeneration the cells also were vacuolated, poorly preserved, and intracellular organisms showed as irregular blue staining masses (Fig. 2).

Smears from the earlier generations were indistinguishable from the 20th generation smears.

Sections of the membranes showed a more constant and progressive picture. At the end of 24 hours after inoculation there were localized lesions characterized by a superficial exudate overlying an area of ectodermal hyperplasia. The superficial ectodermal cells were vacuolated and at times contained large eosinophilic masses. Beneath this area hyperplastic fibroblasts separated the vascular zone from the epithelium. The vessels of the vascular zone were hyperemic and frequently there were areas of hemorrhage in this region. Occasionally a thrombosed capillary was seen. The underlying endodermal cells were hyperplastic and a columnar layer was beginning to differentiate. Forty-eight hours after inoculation (Fig. 6) the zone of fibroblastic growth had widened and become vascularized, the epithelium above it was stratified, and the superficial cells showed hyalinization which fused with the mass of exudate and bacteria. In some membranes there were scattered mononuclear cells in the adjacent mesoderm. These cells had an intense, deep blue staining cytoplasm and nucleolus, and suggested a primitive wandering cell; a few intermediate forms suggested the mesoderm as the origin. Clusters of these cells were occasionally found about vessels. A few membranes showed scattered and perivascular polymorphonuclear leukocytes in the mesoderm. In the dislodged vascular zone there were islands of ectodermal cells. After 48 hours the zone of fibroblastic tissue widened and became less dense. By the 5th day, in some instances, the only evidence of the earlier lesion was a localized area of slightly thickened ectoderm and entoderm and, in the intervening vascular mesoderm, islands of epithelial cells arranged in a semicircle opening toward the ectoderm. In other membranes sometimes as early as the 3rd day, but usually about the 5th day, there appeared an increased perivascular infiltration of polymorphonuclear leukocytes, "primitive cells" and monocytes. This reaction was most notable in, and in many membranes limited to, the area about the

initial lesion. In only a few instances was the epithelial layer of the membrane ulcerated, and only very rarely were there bacteria in the membrane.

Histopathological Studies of the Embryos

One hundred and nineteen embryos of the series were studied. The most constant but probably non-specific finding was a perivascular infiltration found chiefly in the lungs, portal spaces of the liver, skin, pharynx and connective tissue. In a few of the 72 hour infections and in an occasional 96 hour infection this infiltration was present. The infiltrating cells were the "primitive type" and eosinophilic polymorphonuclear leukocytes. In most 5th and 6th day infections there was a perivascular infiltration of variable intensity with eosinophilic polymorphonuclear leukocytes, monocytes and a few lymphocytes. This reaction was less notable in the succeeding days, but in 1 embryo 10 days after inoculation it was present. It was not found in the few hatched chickens that were sectioned. The degree and type of perivascular infiltration in the embryo was similar to that found in the membrane in the region of the initial lesion. Nearly all embryos 4 days or more post-inoculation showed scattered polymorphonuclear leukocytes in the cerebellar meninges. These were rarely seen in uninoculated embryos.

More significant lesions were found in the first 4 embryos sacrificed in the 3rd generation of *16B* series, in 1 embryo of *32M* series, and one of *32MC* series. The embryos of the *16B* series will be described first.

In the brain of the embryo sacrificed 24 hours after inoculation a few small areas of early necrosis were found. In the tissue between the skin and pineal body, in which meninges, dura and connective tissue are not demarcated and in which there is no bone, areas of hemorrhage were found (Fig. 4). In a few of the capillaries in these areas masses of necrotic blood cells were seen and several definite bacilli (Fig. 5) were found both extracellularly and within monocytes. The intracellular forms were shorter and thicker. Heart blood culture was negative.

In the brain of the 48 hour specimen several areas of hemorrhage were found (Figs. 7 and 8). Many of these red blood cells were necrotic and the included capillaries were occluded with necrotic

cells and degenerated endothelial cells. Throughout the brain the capillaries were conspicuously filled with and even appeared occluded by large mononuclear cells. Most of these mononuclear cells were monocytes of the usual type but many were large "primitive type" cells. In the ventricles there was an exudate of these mononuclear cells and polymorphonuclear leukocytes. The meninges and the tissue between the skin and the pineal body were edematous and slightly infiltrated with polymorphonuclear leukocytes and a few monocytes. Some of the monocytes contained degenerated red blood cells. Many of the capillaries in this area had the same appearance as those in the brain substance and in addition a perivascular infiltration with similar mononuclear cells. Scattered throughout the tissues in general in the body and quite prominent in the periportal spaces of the liver were small accumulations of the intense blue staining ("primitive type") monocytes. No bacteria could be positively identified in the sections but the blood culture was positive.

Apparently the above process had progressed to produce a remarkable picture 72 hours after inoculation of the membrane (Fig. 10). Large areas of the brain were necrotic and consisted of a loose mass of red blood cells, brain cells and leukocytes. These necrotic areas had ruptured into the ventricles, which were filled with débris, polymorphonuclear leukocytes, monocytes, red blood cells and numerous pleomorphic bacilli (Fig. 12). There was considerable perivascular infiltration of the meningeal vessels with both types of mononuclear cells and a few polymorphonuclear leukocytes. In the dura were areas densely packed with the intense blue staining mononuclear cells, a few of which contained eosinophilic granules. Small groups of similar cells were found perivascularly in the lungs, portal spaces of the liver, and occasionally in the connective tissue of the skin. In the thoracic and cervical spinal meninges (Fig. 11) an exudate of polymorphonuclear leukocytes and monocytes was found; no bacteria were identified in it. The heart blood culture of this embryo was negative.

The 96 hour specimen showed less extensive areas of necrosis in the brain than the 72 hour, but the process appeared to be essentially the same. The perivascular infiltration of the meningeal vessels was more intense. There was an exudate of polymorpho-

nuclear leukocytes and monocytes in the ventricles, spinal meninges and pericardium; no bacteria were identified positively. The blood culture was positive. All organs showed a perivascular infiltration with monocytes and polymorphonuclear leukocytes.

An embryo of the 1st generation of *32MC*, which was sacrificed 72 hours after inoculation, showed a picture (Fig. 9) similar to that found in *16B* at 72 hours, except that there was not the extensive rupture of necrotic brain tissue into the ventricles; the exudate in the ventricles was chiefly of large monocytes and no bacteria could be demonstrated. The blood culture was negative.

A 2nd embryo taken 5 days after inoculation of the third generation of *32M* showed areas of hemorrhage and necrosis in the brain, an exudate of foamy mononuclear cells in the ventricles, and moderate perivascular infiltration with monocytes and polymorphonuclear cells of the dura, meninges and parenchymatous organs.

The exudate from the membranes of these embryos showed well preserved, numerous intracellular and extracellular organisms; the cells were well preserved and possibly less numerous than usual in *16*. The membranes of the 72 hour (*16B*) (Fig. 6), 96 hour (*16B*) and 5 day (*32M*) were unusual histologically in that each showed a break in the ectoderm by which the bacteria-laden exudate was in communication with the mesoderm. Bacteria were found in the mesoderm in *32M* free beneath the ectoderm, in a localized necrotic area completely walled off from the surrounding mesoderm by fibroblasts in 96 hours (*16B*), and in necrotic exudate in a thrombosed vessel in 72 hours (*16B*).

Survival of the Organisms in the Chorio-Allantoic Membrane and the Blood Stream Invasion of the Embryo

Heart blood cultures from the sacrificed embryos showed no constant time of invasion or of disappearance of organisms from the blood stream. Each strain gave at least 1 positive blood culture. No hatched chickens gave a positive blood culture. In all strains except *P* the membrane continued infected through the 4th day; several thereafter became sterile. The membrane of a hatching chicken of Series *15* was positive.

TABLE I

Membrane and Heart's Blood Cultures of Different Series of Inoculated Embryos

Strain	Generation	Culture	24 hours	48 hours	72 hours	4 days	5 days	6 days	7 days	8 days	9 days	10 days
16B	3	M	++	+	+	+	−	+				
		HB	−	+	−	+	−	−				
32M	5	M	++	+	+	+	+	+				
		HB	−	−	+	−	−	−				
32P	1	M	++	++	+	+						
		HB	−	+	+	−						
32MC	1	M	+++	+++	++	−		+				
		HB	+	+	−	−		−				
37	1	M	++	++		++	−					
		HB	−	−		+	−					
55	2	M	+++	+++	+++	+	+					
		HB	−	−	−	+	−					
P	20	M	++	++		−	+	−	−	+		
		HB	−	+		−	−	−	−	−		
14	20	M	++	+++	++	+	+	−	+			
		HB	+	+	−	+	+	+	−			
15	20	M	+++	+++	++	+	−					
		HB	+	−	−	+	−					
16	20	M	+++	+++	+	+++	+					
		HB	+++	++	+	−	−					
32M	20	M	+++	+++	++	++	+	+	−		−	+
		HB	+++	+	−	−	−	−	−		−	−
32P	20	M	+++	+++		−	+++	−	+	++	−	+
		HB	−	+		+	+	−	−	−	−	−
32MC	20	M	+++	+++	+	+++	+++	+++	+++	−	+	
		HB	+++	++	+	−	−	−	+	−	−	

HB = Heart blood culture on Levinthal agar.
M = Membrane culture on Levinthal agar.
+ to +++ = Relative number of colonies.
− = Negative culture.

Survival of the Embryos After Inoculation of the Chorio-Allantoic Membranes

The survival period varied considerably for the same strain. After the first 3 generations of *16* the embryos of that series, with occasional exceptions, died within 48 hours; 1 of the embryos hatched. None of Series *14* survived the 9th day after inoculation, but many survived 5 days. Several of Series *P* and *14* survived 11 days after inoculation and 1 of each hatched. The embryos of Strains *32* had on the whole the longest survival period; 3 of the *32MC* and 3 of the *32M* hatched.

Immunological Studies of the Blood of Chickens Hatched From the Inoculated Series

These chickens were sacrificed and the serum used for agglutination tests against the strain of organisms inoculated on the membrane. Thread and ordinary agglutination reactions were negative. The precipitative reactions were negative.

Discussion

This work has been reported in detail in order to help further investigation in a relatively new field, rather than for any significance in itself. Now that it has been found by chance that a lesion can be produced by *H. influenzae,* which is in many respects a counterpart of meningitis and ependymitis in children (Fig. 13), although the lesion has not yet been reproduced at will, the factors determining this initial infection become of primary interest. The chick embryo offers unusual possibilities in the investigation of such a problem.

From this investigation no conclusions can be drawn concerning the rôle or relations of any of the variable factors — host resistance, host susceptibility, virulence and quantity of organisms, duration of infection or availability of the organisms to susceptible tissues — in the establishment of the pathological process. That there may be a variation in virulence which may be an important factor is suggested by the fact that the 4 significant embryos of *16B* came from the same generation, whereas embryos from several other generations of *16* showed no encephalitis or meningitis. The strain used in starting *16B* had all the characteristics, as de-

HB = Heart blood culture on Levinthal agar.
M = Meningeal culture on Levinthal agar.
+ to +++ = Relative number of colonies.
± = Negative culture.

termined by the usual methods, that the original strain and the 20th generation strain possessed. Our present methods of determining virulence, however, are admittedly inadequate. Only 1 embryo was sacrificed from the generation of *32M* showing encephalitis. That the virulence of the organisms is the only determining factor is contradicted by the fact that the embryo taken 6 days after inoculation in the same generation of the *16B* series, and the other 5 taken from the *32MC* series, showed no evidence of encephalitis or meningitis. In the membranes of 3 out of the 6 significant embryos there was a break in the epithelium and bacteria were found within the membrane. That a source of continued infection is necessary and is obtained by some such process is suggested. However, the invasion of the membrane may be a reflection of the virulence of the organism or the general susceptibility of the host. In only 3 other membranes was a break in the epithelial barrier noted; these embryos were not remarkable. The numerous positive blood cultures in embryos showing no encephalitis eliminate a bacteriemia as the sole determining factor. The reaction in the tissues between the skin and the brain in the early stages of infection (*16B* 24 hours and *16B* 48 hours) may be of significance and immediately suggests the anatomical difference between infants, who are susceptible to influenza meningitis, and adults, who are not. Cultures of the amniotic fluid were not made and further investigation of this as a source of infection is indicated. Experiments with *H. pertussis* suggest that ready accessibility of the microorganism to the susceptible tissues is a very important factor, and that altering the route of inoculation may yield more constant infections.

The cause of the perivascular reaction in the later stages of infection of most of the embryos is not clear. No organisms were ever demonstrable in these areas. The early response with the intense blue staining mononuclears may represent a hyperplasia rather than an infiltration. According to the blood culture findings, the perivascular reaction does not depend on a continued bacteriemia. The possibility of circulating toxins is to be considered. The continued bacterial growth in the initial lesion of the membrane in some instances might be a source of toxin production; however, the perivascular reaction was sometimes found when the membranes had become sterile. One embryo, which showed con-

siderable perivascular reaction and had a sterile culture of the blood and epithelial surface of the membrane, showed organisms adherent to the endoderm. Cultures of the allantoic fluid were not taken but growth in this extensive medium must be considered in explaining the perivascular reaction.

It is hoped that by redirected and further investigation infection of the brain and meninges can be consistently produced. When this is accomplished a comparative study of the various strains and the effect of successive transfers may be effectively undertaken. Also, and of more immediate significance, immunological studies can then be started.

Summary

A detailed account of the study of *H. influenzae* infection by the use of the chick embryo is given. In the course of the study a few of the embryos were found to have an encephalitis and meningitis. Investigations to determine the factors which establish this infection with *H. influenzae* and by which these lesions will be consistently produced are indicated.

REFERENCES

1. Goodpasture, E. W. Use of the embryo chick in investigation of certain pathological problems. *Southern M. J.*, 1933, **26**, 418–420.

2. Goodpasture, Ernest W., and Anderson, Katherine. The problem of infection as presented by bacterial invasion of the chorio-allantoic membrane of chick embryos. *Am. J. Path.*, 1937, **13**, 149–174.

3. Buddingh, G. John, and Polk, Alice. Meningococcus infection of the chick embryo. *Science*, 1937, **86**, 20–21.

4. Pittman, Margaret. Variation and type specificity in the bacterial species *Hemophilus influenzae*. *J. Exper. Med.*, 1931, **53**, 471–492.

5. Fothergill, LeRoy D., and Chandler, Caroline A. Observations on the dissociation of meningitic strains of *H. influenzae*. *J. Immunol.*, 1936, **31**, 401–415.

6. Goodpasture, Ernest W., and Buddingh, G. John. The preparation of antismallpox vaccine by culture of the virus in the chorio-allantoic membrane of chick embryos, and its use in human immunization. *Am. J. Hyg.*, 1935, **21**, 319–360.

DESCRIPTION OF PLATES

PLATE 135

FIG. 1. Egg showing chorio-allantoic membrane with purulent exudate. *H. influenzae* 48 hours.

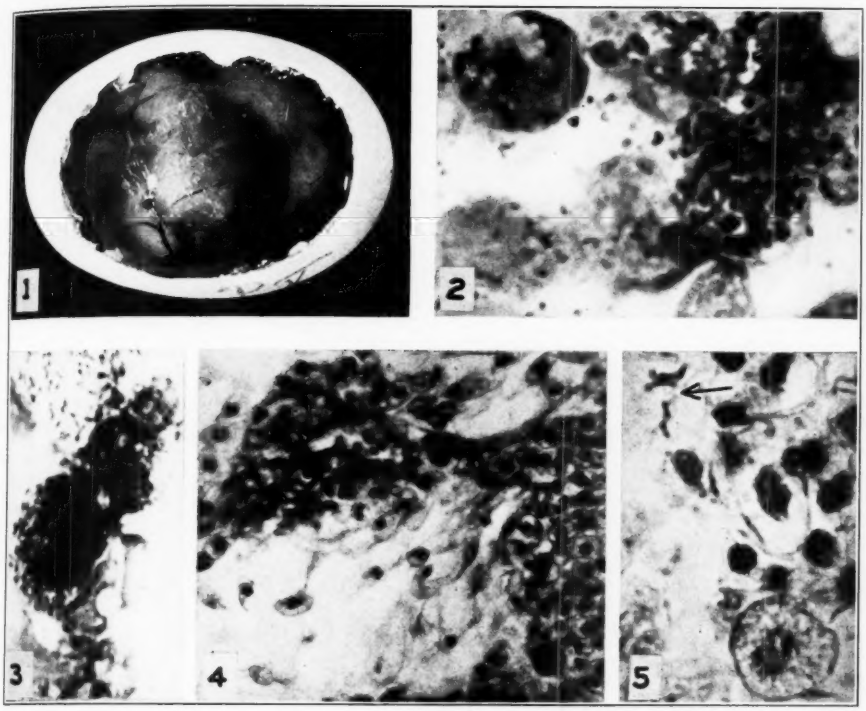
FIG. 2. Membranal exudate. Degenerated cells and intracellular and extracellular bacilli (dark masses). × 1600.

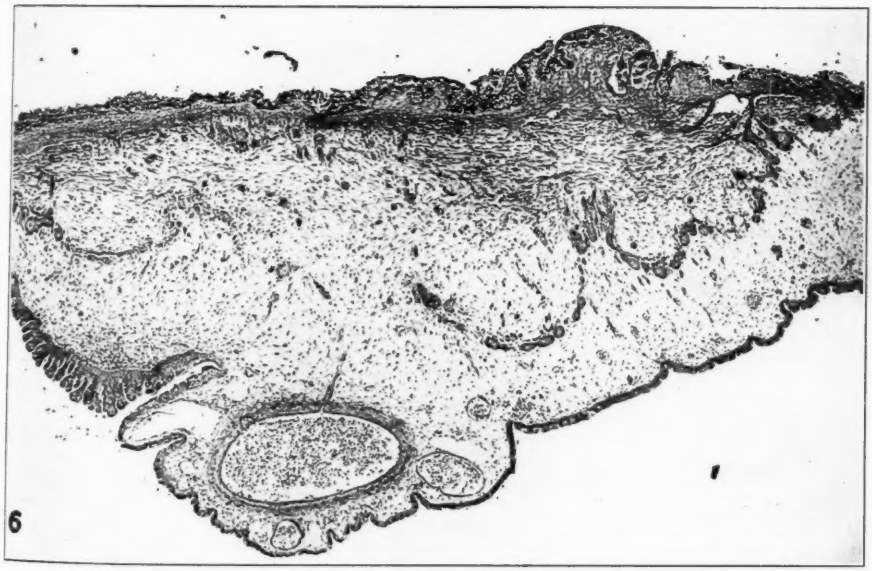
FIG. 3. Membranal exudate. Well preserved intracellular and extracellular bacilli. × 1600.

FIG. 4. Hemorrhage and necrosis of blood cells. × 550.

FIG. 5. Lower right section of Fig. 4 enlarged to show bacilli. × 1400.

FIG. 6. Chorio-allantoic membrane; *H. influenzae* 48 hours. × 55.





Gallavan

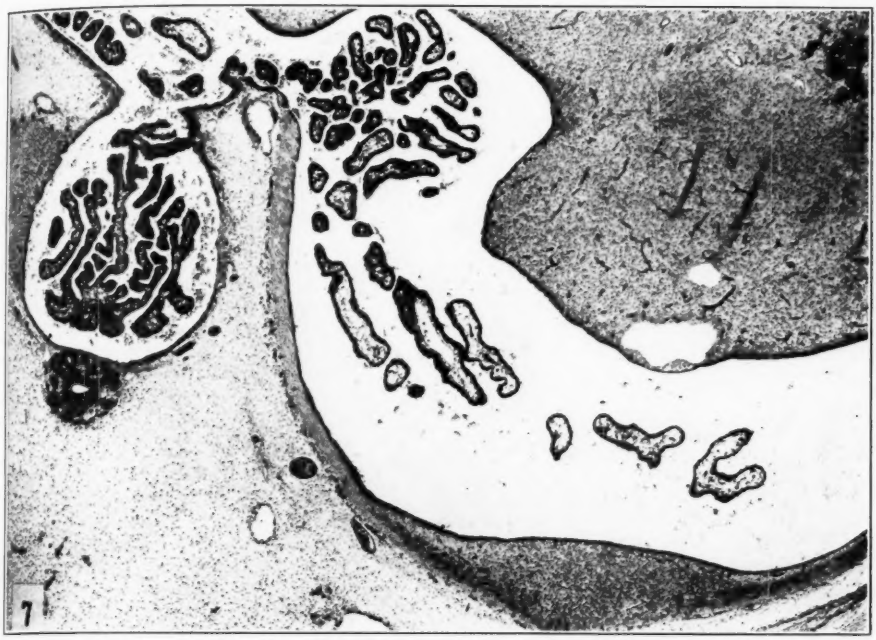
Encephalitis and Meningitis in Chick Embryo

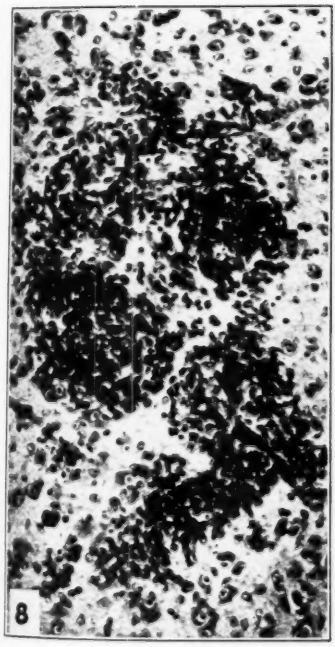
PLATE 136

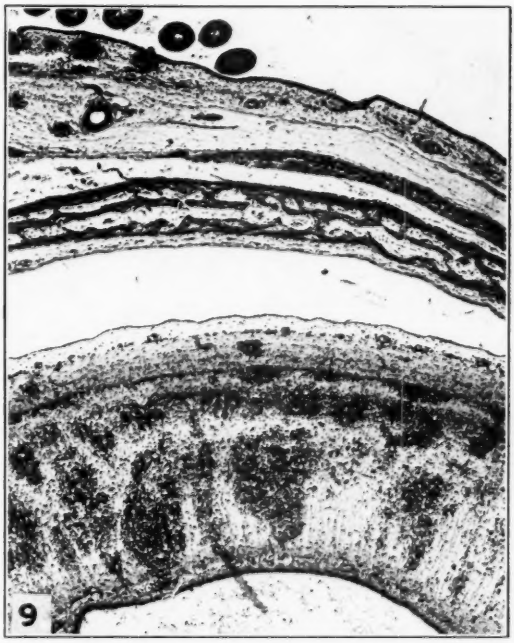
FIG. 7. Brain of chick embryo 48 hours postinoculation of membrane with *H. influenzae 16B*. Note area of hemorrhage in upper right corner, conspicuous capillaries in the brain represented by the dark branching structures, dilated ventricles containing cellular exudate and cellular infiltration of the connective tissue. × 30.

FIG. 8. Area of hemorrhage and necrosis of blood cells seen in Fig 7. × 200.

FIG. 9. Brain of embryo 72 hours postinoculation of membrane with *H. influenzae 32MC*. × 44.







Gallavan

Encephalitis and Meningitis in Chick Embryo

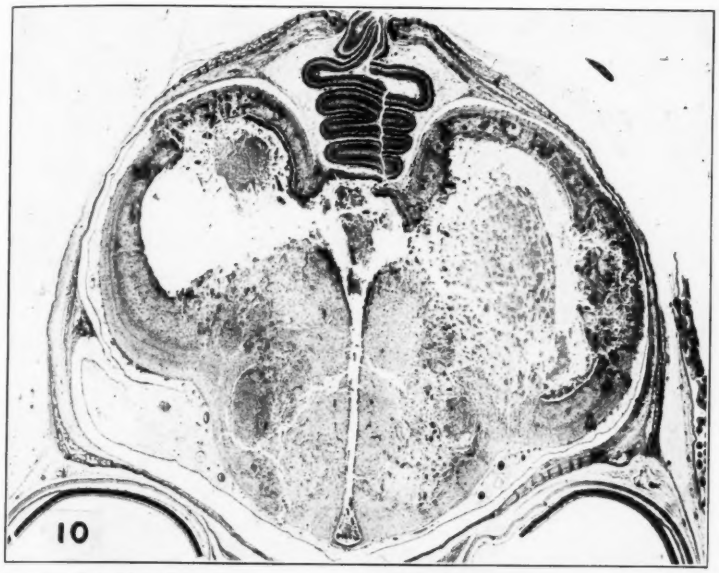
PLATE 137

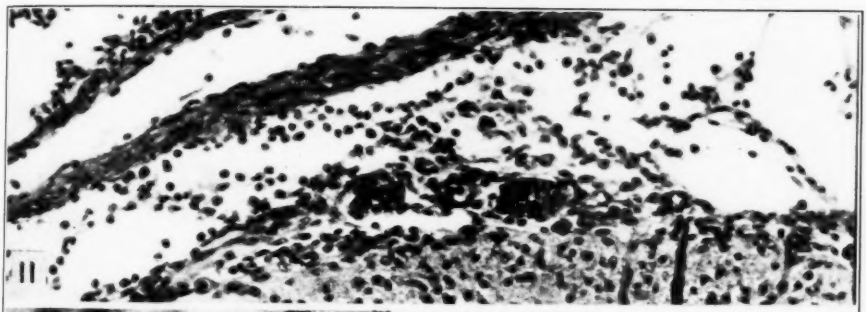
FIG. 10. Brain of embryo 72 hours postinoculation of membrane with *H. influenzae 16B*. × 9.

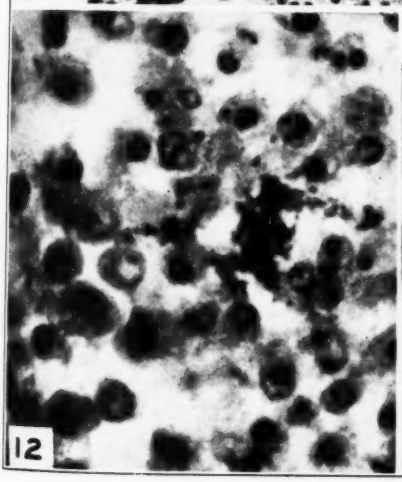
FIG. 11. Exudate in spinal meninges of chick embryo. × 190.

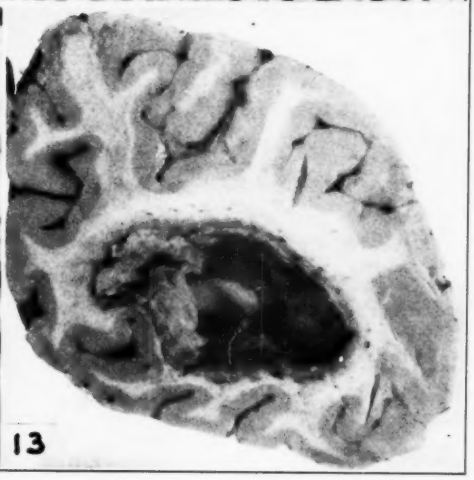
FIG. 12. Exudate shown in ventricle in Fig. 10. Irregular black mass represents a clump of bacilli. × 1200.

FIG. 13. Gross section of brain of a child 2 years of age showing suppurative ependymitis, encephalitis, meningitis and hydrocephalus.









Gallavan

Encephalitis and Meningitis in Chick Embryo

INFECTION OF CHICK EMBRYOS WITH H. PERTUSSIS REPRODUCING PULMONARY LESIONS OF WHOOPING COUGH *

MAE GALLAVAN, M.D., AND E. W. GOODPASTURE, M.D.

(From the Department of Pathology, Vanderbilt University Medical School, Nashville, Tenn.)

The rôle of *Haemophilus pertussis* in the production of pulmonary lesions of human whooping cough has been the subject of much dispute. The following experiments throw some new light on this subject and indicate the method by which a more extensive study of *H. pertussis* infection in relation to the etiology and pathogenesis of whooping cough is being carried on.

As a preliminary to the study of experimental infection with *H. pertussis* the microscopic sections of 11 autopsy cases on record at Vanderbilt Hospital were reviewed.

In addition to the presence of Gram-negative bacilli between the cilia, the inflammatory reaction in and about the trachea, bronchi and bronchioles, and the bronchopneumonia, we were impressed by the presence of a lesion in the respiratory epithelium, especially that of the bronchi and bronchioles, which has attracted little or no attention.

Arnheim [1] mentioned desquamation of ciliated epithelium in whooping cough and described small bacilli on these cells. Mallory and Horner [2] gave a more detailed description of the peculiar relation of the bacilli to the ciliated epithelium in 3 cases, but stated that they could find no evidence of epithelial necrosis, nor did they describe any degenerative changes in these cells.

The studies of Feyrter [3] tend to confirm the impression of Posposchill that the lung is the seat of the essential lesions of whooping cough. In describing the respiratory epithelium he cites inflammatory infiltration, sometimes with miliary abscess formation, in the bronchial epithelial layer, even rupturing into the lumen and leaving small ulcers.

In our study of human material it appeared also that the severest lesions, in association with the bacilli situated in the characteristic interciliary position or on the partially deciliated border, were in

* Aided by grants from the Josiah Macy, Jr. Foundation and the Division of Medical Sciences, Rockefeller Foundation.

Received for publication July 10, 1937.

the bronchi and bronchioles, and to a less extent in the trachea and the nasopharyngeal ciliated epithelium (studied in 1 case).

The inflammatory response to the presence of the bacilli is no doubt due to injury induced by these specific microorganisms, although the nature of that injury has not been revealed by previous work. In our cases the injury appears to be for the most part manifested in the appearance of the respiratory epithelial layer usually at points surmounted by a zone of interciliary bacilli. This injury is relatively mild but may induce necrosis. The cells most affected, as indicated by their cytology, are situated in the midzonal or basal layers of the epithelial covering of bronchi and bronchioles. The superficial ciliated cells are relatively intact. Degeneration and even necrosis of the deeper epithelial cells may be demonstrated, and the inflammatory reaction, polymorphonuclear leukocytes, macrophages (some of which appear to be derived from epithelium) and mitotic figures correspond in intensity to the degenerative lesion — that is to say, it is most pronounced in the midzonal and basal layer. The epithelium is in places elevated from its basement membrane by exudate, and miliary abscesses (foci of polymorphonuclears and macrophages) frequently are limited to the midzonal layer.

The situation of this chief injury and inflammatory reaction in the midzonal and basal layers of the bronchial epithelium is quite characteristic. The type of lesion suggests the action of a specific toxic agent. This type of lesion was not found in the cases of interstitial bronchopneumonia associated with influenza and measles which were examined. Whether or not the associated bronchopneumonia may be caused by *H. pertussis* is as yet not determined for the human disease.

It is evident that further study of the human lesion is necessary for the establishment of the pathogenesis of whooping cough, but there can be no doubt that a specific lesion exists.

It would be highly desirable to have at hand a suitable susceptible experimental animal in which a more thorough study of the etiology and pathogenesis of this important disease could be carried out. None of the usual laboratory animals, with the possible exception of some monkeys and the chimpanzee, appear to be susceptible to the infection.

Although most investigators seem to believe that *H. pertussis*

is the chief or sole inciting agent, there are others who think that an undetermined virus may be an essential preliminary invader.

In the investigation of *H. pertussis* infection in chick embryos to be described, we believe the essential lesion of whooping cough has been reproduced, and it is hoped that the method employed may serve as a means of throwing additional light on the etiology, pathogenesis and immunology of this specific infection.

Experimental

The first part of the experimental investigation of *H. pertussis* was started at the same time and undertaken in the same manner as that of *H. influenzae* reported in the present issue of this journal.

Only one strain of *H. pertussis* was studied, and no clinical cases or autopsy material were available during the investigation. The strain used, isolated by Dr. John Toomey, Oct. 24, 1936 and kindly sent to us by him, had been transferred a number of times on Bordet-Gengou medium containing 25 per cent human blood, and then several times on Difco Bordet-Gengou agar with 25 per cent defibrinated rabbits' blood. The latter medium was used throughout the experiment.

This strain of *H. pertussis* had the following characteristics: morphologically it was a small Gram-negative bacillus which grew slowly and produced hemolysis on Bordet-Gengou medium; it did not reduce nitrates, ferment sugars, or produce indol. A 2 billion saline suspension of a 48 hour culture was agglutinated by pertussis-agglutinating serum (Lilly) through dilutions of 1:5120. Three mice inoculated intraperitoneally with 0.5 cc. of a saline suspension of 2 mg. of wet organisms from a 72 hour culture died within 24 hours. One tenth cc. of this suspension injected intradermally into a rabbit produced central necrosis surrounded by a purplish red indurated area that persisted for 10 days.

For the first experimental series a loopful of a 20 hour culture was transferred to the chorio-allantoic membrane of 6 11 day chick embryos, and from this the organisms were carried through 20 consecutive generations on the chick membranes. Embryos were taken for microscopic study from the first 3 and the 20th generations.

The bacilli from the 19th generation eggs grew more rapidly on Bordet-Gengou medium and agglutinated through only 1:160

serum dilution. Only 1 of 3 inoculated mice died within 24 hours. The remaining 2 died within 48 hours. In other respects the characteristics of the organisms as originally determined were unchanged. A control culture of bacilli transferred through 20 generations on Bordet-Gengou medium was agglutinated through a 1:1280 serum dilution.

Membrane and heart's blood cultures were not taken on the embryos sacrificed in the first 3 generations. In the 20th generation the 4th and 5th day postinoculation specimens showed 1 colony each from the blood culture; the 6th day specimen gave many colonies. The cultures of the membranes were positive through the 7 days of survival.

The Chorio-Allantoic Lesion

In the gross the chorio-allantoic membranes 24 hours after infection usually showed a moderate edema, considerable hemorrhage and purulent exudate (Fig. 1). The appearance did not change greatly until the membrane began to dry on the 4th or 5th day postinoculation. The older membranes were considerably thickened when removed.

Smears made from the membranal exudate showed bacilli, red blood cells, polymorphonuclear leukocytes and monocytes. The bacilli were well preserved, small, and only rarely showed pleomorphism with chain formation. A few enlarged bacilli were scattered in the polymorphonuclear leukocytes and less frequently in the monocytes. Rarely a well stained mass of small bacilli was found in these cells. Eosinophilic polymorphonuclear cells predominated but the monocytes tended to increase after 48 hours. Degenerated cells were rarely found.

The microscopic examination of several membranes revealed a striking picture: 24 hours after inoculation (Fig. 2) areas of necrosis were seen in the hyperplastic ectodermal epithelium and in the adjacent vascular tissue, and fragmented nuclei were numerous and conspicuous. Throughout the edematous membrane were scattered numerous eosinophilic polymorphonuclear leukocytes and small foci of these leukocytes were frequent in the mesoderm and necrotic epithelium. In some areas the mesodermal cells showed a marked increase in size and intensity of staining, and a slight increase in number; this was especially notable about the blood ves-

sels where a deeply staining fibrous tissue resulted. At times areas of this thickened vascular wall were necrotic and infiltrated with polymorphonuclear leukocytes. There were large areas of hemorrhage containing masses of necrotic cells and the endothelium of the involved capillaries was degenerated. The endoderm was hyperplastic. No bacteria could be identified among the numerous nuclear fragments. Apparently this process had progressed until, within 48 hours after inoculation, hyperplasia of the mesodermal cells in an edematous tissue had produced a picture suggesting dense alveolar tissue, in the alveoli of which were numerous polymorphonuclear leukocytes. In the 72 hour membrane the tissue was more compact. The interstices between the deeply staining bands of hyperplastic fibrous tissue contained an eosinophilic fluid, a few well preserved leukocytes and fragments of degenerated cells. Necrosis of the tissue was not as prominent as in the earlier stages. The membrane from the 20th generation embryo 6 days postinoculation showed a deeply ulcerated area extending into the mesoderm and filled with masses of bacilli (Fig. 3). The 72 hour 3rd generation, and the 24 hour and 48 hour 20th generation membranes did not show the above described lesions, but appearances similar to *H. influenzae* infection. After 72 hours the membranes showed edema, fibrous tissue and large foci of intense blue staining mononuclear cells with prominent nucleoli and occasional foci of polymorphonuclear leukocytes. These membranes also were not markedly different from those of *H. influenzae*.

Thirteen embryos were taken for microscopic study, 6 from the earlier generations, representing infections of from 1 to 7 days, and 7, representing infections through 7 days, from the 20th generation of the organisms on eggs.

In the 24 hour, 48 hour, and 96 hour specimens from both groups there were scattered throughout the tissues small areas of hemorrhage in which necrotic red blood cells and an occasional thrombosed capillary were present. There was no associated cellular reaction or bacteria. Except for necrosis in the areas of hemorrhage, the endothelial cells appeared normal. In the older embryos there was the non-specific perivascular infiltration with polymorphonuclear leukocytes and monocytes similar to that found in *H. influenzae* infection.

Pulmonary Lesions

Two embryos of the foregoing series were remarkable. Numerous tiny bacilli were seen in the occasional ciliated epithelial cells of the esophagus (Fig. 12) in the 3rd generation embryo taken 5 days after inoculation of the membrane. There was no necrosis or cellular reaction of the underlying cells. No definitely ciliated epithelium could be found in the respiratory tract. An embryo of the 20th generation, 6 days after inoculation, showed a significant pathological picture in the lungs (Fig. 5). The cilia of the epithelial cells of the bronchi and air sacs enmeshed myriads of tiny deeply staining bacilli (Figs. 6, 7, 9, 10). In the midzonal and basilar portions of this ciliated epithelium were areas of granular necrosis containing many deeply basophilic fragments. At this stage only an occasional monocyte or leukocyte was seen infiltrating these areas. The muscularis appeared intact and a few monocytes and polymorphonuclear leukocytes were present in the peribronchial tissue. The bronchial lumen contained many polymorphonuclear leukocytes, monocytes, amorphous eosinophilic material, and occasionally a few extracellular and intracellular degenerated bacteria. In this embryo no well defined cilia were found in the pharynx or trachea, and in these situations the epithelial cells showed no necrosis. The non-ciliated bronchiolar epithelium was well preserved. Some of the bronchioles contained a dense occluding mass of leukocytes and degenerated bacteria. The alveolar epithelium, where preserved, was composed of large, pale ragged cells which in many areas were desquamated. The alveoli contained these desquamated cells, polymorphonuclear leukocytes, monocytes, and in several there were markedly degenerated bacteria. The interalveolar tissue was hyperplastic, the nuclei of the cells enlarged, and the fibrils thickened and deeply staining. There was an abundant interstitial infiltration of monocytes and polymorphonuclear leukocytes. In many situations where the alveolar epithelium was desquamated and the alveoli compressed by the thickened walls, the tissue appeared as an inseparable mass of fibroblasts, monocytes and polymorphonuclear leukocytes. A few areas of hemorrhage were present. Altogether the lesion constituted a diffuse bronchitis and bronchopneumonia involving the entire lung. The ciliated epithelium of the esophagus contained many bacteria but there was no evidence of necrosis.

In an attempt to reproduce this lesion in the lung, a 2nd series of 4 groups of embryos was inoculated. The 19th generation egg culture from the 1st series was used to initiate infection. It had been maintained through 4 transfers for 2 months on Bordet-Gengou medium.

Ten 13 day embryos were inoculated into one of the larger veins of the chorio-allantoic membrane with 0.05 to 0.1 cc. of a 1 billion per cc. saline suspension of a 36 hour culture. Two of these embryos were sacrificed at the end of 4 days and the 2 still living at 6 days were killed for study. Cultures taken from the mouth and heart's blood were negative and the microscopic study revealed nothing remarkable.

Ten day embryos were inoculated by forcing a needle directly into the body and introducing with a syringe 0.1 cc. of a 15 billion per cc. saline suspension of a 12 hour culture. Two of these survived to be autopsied 6 days later. The mouth cultures on both were positive and 1 had a positive blood culture. On microscopic examination no organisms or definitely ciliated cells could be found in the esophagus or respiratory tract.

The amniotic sac of 7 13 day embryos was inoculated with 0.1 cc. of a saline suspension of a 36 hour culture, 1 billion per cc. Two of these embryos were sacrificed at the end of 4 days. Microscopic examination showed nothing significant. One had definite ciliated cells but the mouth and blood cultures were negative. The other, which showed no ciliated epithelial cells, had a positive mouth culture and negative blood culture. Two embryos of this group survived to be sacrificed for study at 6 days. The mouth cultures on each showed numerous colonies; the blood cultures were negative. Microscopically each embryo had numerous well preserved bacteria on the ciliated epithelium of the pharynx, trachea, bronchi, air sacs and esophagus. One embryo showed no apparent local reaction to this infection. The 2nd embryo showed only very rarely an early granular necrosis of the epithelium and a slight infiltration of the submucosa with polymorphonuclear leukocytes. A few secondary bronchi, bronchioles and alveoli contained a moderate number of monocytes, polymorphonuclear leukocytes and degenerated bacteria. Some groups of terminal alveoli contained desquamated alveolar cells and occasional degenerated bacteria, polymorphonuclear leukocytes and monocytes.

In these areas the alveolar walls were poorly defined and the interstitial tissue showed a few monocytes and polymorphonuclear leukocytes; some interstitial cells were hyperplastic and others appeared compressed, having a distorted deeply staining nucleus.

A 4th group, consisting of 9 12 day embryos, was inoculated in the same manner as the 3rd group, using a 15 billion suspension of a 12 hour culture. Only 1 of these embryos was alive on the 4th day; it was then sacrificed. The mouth and heart cultures were positive. Microscopically the epithelial cells of the esophagus showed well defined cilia and only in these situations were organisms found.

These experiments thus far indicate that the method of choice for successful pulmonary infection is by way of the amniotic fluid by means of which the bacilli are brought, probably by respiratory movements, into direct contact with ciliated epithelium, which seems to be the best medium of the host for stimulating their growth. Occasional infection of the lungs, however, may occur from primary infection of the chorio-allantoic membrane.

Discussion

There is excellent clinical evidence of the experimental production of whooping cough in animals [4-8] and in man [9] by the introduction of *H. pertussis* into the respiratory tract. The pathological lesions have been investigated in only a few of these animals. Sauer and Hambrecht [10] autopsied two of their monkeys during the experimental whooping cough infection. They described bacilli in the cilia of respiratory epithelium, and peribronchial inflammation, but did not find the midzonal necrosis and leukocytic infiltration that we have found in cases showing peribronchitis to the extent described in their Monkey A 5. However, the monkeys were sacrificed and a terminal picture was not to be expected. Shibley [6] found no bacteria in the cilia of his autopsied chimpanzee.

That *H. pertussis* causes the bronchial infiltration in whooping cough has been taken for granted except when this infiltration has been attributed tentatively to the action of an undemonstrated virus. That the pneumonia is associated specifically with *H. pertussis* infection has been concluded by Smith,[11] Fonteyne [12] and others; and that *H. pertussis* can produce an interstitial pneu-

monia has been indicated by Sprunt, Martin and Williams.[13] However, it has never been unquestionably established that *H. pertussis* alone can produce the lesions found in man and experimental animals with whooping cough. This proof has been lacking largely because it is impossible to rule out other associated or secondary infections in man and animals.

The chick embryo offers an unparalleled opportunity to study the pathological process of a pure infection. The accessibility of the respiratory tract through the amniotic fluid aids greatly in the investigation of an infection by an organism to which the respiratory tissues are susceptible.

By this investigation it has been established that the chick embryo is susceptible to infection with *H. pertussis* and that the lesions found in fatal cases of human whooping cough can be produced in detail in the chick embryo lung by *H. pertussis* alone. These facts indicate that *H. pertussis* is the inciting cause of whooping cough in man, for in these experiments it cannot be assumed with reason that the pulmonary infection was initiated by an associated virus or by any other microorganism.

The experiments recorded show that *H. pertussis* tends to localize specifically on and in the ciliated border of respiratory and esophageal epithelium. There is no other tissue in this host, removed from the primary infection in the chorio-allantoic membrane, where evidences of metastatic infection have been found. Although the bacilli may rarely infect the lung from the primary membranal lesion, experiments thus far indicate that their admission to the lung through amniotic fluid is a preferable route.

There seems to exist a specific reproductive relation between the ciliated border of epithelium of the respiratory tract and the bacilli; and the epithelium of the bronchi and bronchioles appears to be more advantageous for the growth of this bacterium than that of the trachea and elsewhere.

The growth of the bacilli in the ciliated border is usually associated with an injury, leading to necrosis, of epithelial cells situated in the midzonal and basal layers of the bronchial and bronchiolar epithelium. This injury is responded to by an exudate of fluid, polymorphonuclear and mononuclear leukocytes within the epithelial layer, in the lumens, and in peribronchial and interstitial tissues of the lung in general. The pulmonary alveoli may be filled

with cellular exudate, desquamated cells, fluid and degenerating bacteria. There is as yet no definite evidence that the bacilli grow in the lung except in association with the ciliated border of epithelium. From these sites it is probable they are borne by respiratory movements to the alveoli where they degenerate, but they may induce here also degeneration and necrosis of non-ciliated respiratory epithelium.

In the chick embryo, therefore, the lesions indicate that the growing *H. pertussis* liberate an injurious substance which acts primarily on non-ciliated respiratory epithelium, causing degeneration and necrosis; and *H. pertussis* in this bird likewise can cause bronchopneumonia, in addition to the inflammatory reaction in and about the larger air channels.

In sections of widely expanded air sacs connected with bronchi it is possible to find isolated islands of ciliated epithelium containing abundant growth of bacilli, while the surrounding non-ciliated epithelium contains none (Fig. 11). Under these circumstances it is only beneath the ciliated infected cells that necrosis of epithelium occurs. This indicates quite clearly that the injurious effect is the result of some toxic product of the growing bacilli acting locally.

Rather generalized perivascular infiltration in the embryo may be interpreted as an indication of a more widespread effect of such an injurious substance or substances.

This is the report of preliminary work. Further investigations along many lines are suggested. By this experimental approach it may be possible to determine the relation of the factors that establish the infection and to study the progress of the lesions.

The importance of the ready access of the microorganism to the susceptible tissue is indicated by the experimental record. That a particular type of cell may be of significance in the pathogenesis of an infection has been indicated elsewhere,[14] and the suggestion has been made that there might be a reproductive relation between *H. pertussis* and the ciliated respiratory epithelium.[15] That such a relation exists is strongly indicated in these studies. Although it is difficult to be certain of the absence of ciliated epithelium in cells with a cuticular margin overlaid with eosinophilic fluid and amorphous material, in a large series of embryos definitely ciliated respiratory epithelium was not found before the 15th day and

usually not until the 17th day. The few ciliated esophageal cells usually have well defined cilia by the 14th or 15th day.

Possibly the variation in the picture produced by the infection of the 2 embryos of the same age given equivalent amounts of the same suspensions is due to a variation in the time of development of the ciliated epithelium, or to the relative position of the embryo in the amniotic cavity, or to the establishment of or a variation in the respiratory movements. This latter possibility is suggested by the work of Snyder and Rosenfeld [16] on fetal respirations.

Phenomena involved in the specific localization of bacteria in the infected host are susceptible of investigation by the method used.

Summary and Conclusions

Chick embryos are susceptible to infection with *H. pertussis*. Infection of the respiratory tract, including the lungs of the embryo, has been induced by infection of the chorio-allantoic membrane and by inoculation of the amniotic fluid with *H. pertussis*.

The pulmonary lesions of human whooping cough are reproduced in detail by the pulmonary infection of the embryo. These are characterized by growth of *H. pertussis* on the ciliated border of respiratory (and other) ciliated epithelia, necrosis and inflammation of the middle and basal layers of the epithelial membrane, intrabronchial and peribronchial cellular exudation, and alveolar and interstitial pneumonia.

These experiments indicate that *H. pertussis* alone is the inciting cause of whooping cough.

References

1. Arnheim, G. Zur Pathogenese des Keuchhustens. *Berl. klin. Wchnschr.*, 1903, **40**, 665–667.
2. Mallory, F. B., and Horner, A. A. Pertussis: the histological lesion in the respiratory tract. *J. M. Research*, 1912–13, **27**, 115–123.
3. Feyrter, F. Ueber die pathologische Anatomie der Lungenveränderungen beim Keuchhusten. *Frankfurt. Ztschr. f. Path.*, 1927, **35**, 213–255.
4. Inaba, I. Über den Bordet-Gengou'schen Keuchhustenbacillus bes. Uebertragungsversuche des Keuchhustens auf Tiere. *Ztschr. f. Kinderh.*, Orig., 1912, **4**, 252–264.
5. Sauer, Louis W., and Hambrecht, Leonora. Experimental whooping cough. *Am. J. Dis. Child.*, 1929, **37**, 732–744.

6. Shibley, G. S. Etiology of whooping cough. *Proc. Soc. Exper. Biol. & Med.*, 1934, **31**, 576–579.

7. Culotta, Charles S., Harvey, Daniel F., and Gordon, Ernest F. Whooping cough. II. Experimental study. *J. Pediatrics*, 1935, **6**, 743–752.

8. Rich, A. R., Long, P. H., Brown, J. H., Bliss, E. A., and Holt, L. E., Jr. The experimental production of whooping cough in chimpanzees. *Bull. Johns Hopkins Hosp.*, 1936, **58**, 286.

9. MacDonald, H., and MacDonald, E. J. Experimental pertussis. *J. Infect. Dis.*, 1933, **53**, 328–330.

10. Sauer, Louis W., and Hambrecht, Leonora. Whooping cough: the site of the lesion. *Arch. Path.*, 1929, **8**, 944–953.

11. Smith, Lawrence W. The pathologic anatomy of pertussis with especial reference to pneumonia caused by the pertussis bacillus. *Arch. Path.*, 1927, **4**, 732–742.

12. Fonteyne, P. Contribution à l'étude du pouvoir pathogène du bacille coqueluchеux. *Compt. rend. Soc. de biol.*, 1932, **110**, 976–978.

13. Sprunt, Douglas H., Martin, Donald S., and Williams, Jarrett E. Interstitial bronchopneumonia. II. Production of interstitial mononuclear pneumonia by the Bordet-Gengou bacillus. *J. Exper. Med.*, 1935, **62**, 449–456.

14. Goodpasture, Ernest W. Concerning the pathogenesis of typhoid fever. *Am. J. Path.*, 1937, **13**, 175–185.

15. Goodpasture, Ernest W. Intracellular parasitism and the cytotropism of viruses. *Southern M. J.*, 1936, **29**, 297–303.

16. Snyder, F. F., and Rosenfeld, M. Breathing of amniotic fluid as normal function of fetal respiration. *Proc. Soc. Exper. Biol. & Med.*, 1937, **36**, 45–46.

DESCRIPTION OF PLATES

PLATE 138

FIG. 1. Lesion on chorio-allantoic membrane 48 hours after inoculation with *H. pertussis*.

FIG. 2. Section of chorio-allantoic membrane 24 hours after inoculation. Edema and polymorphonuclear reaction especially evident. × 650.

FIG. 3. Lesion on membrane showing necrosis. Rarely so destructive. × 55.

FIG. 4. Lung of normal embryo to be compared with Fig. 5. × 30.

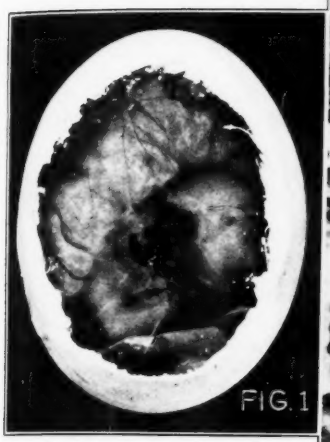


FIG. 1

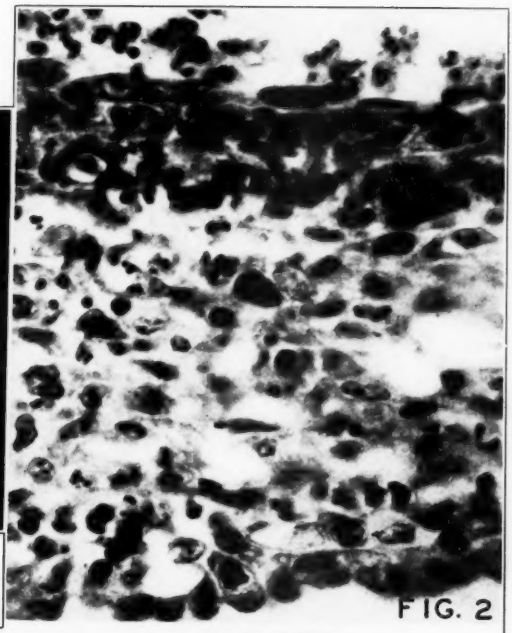


FIG. 2

FIG. 3

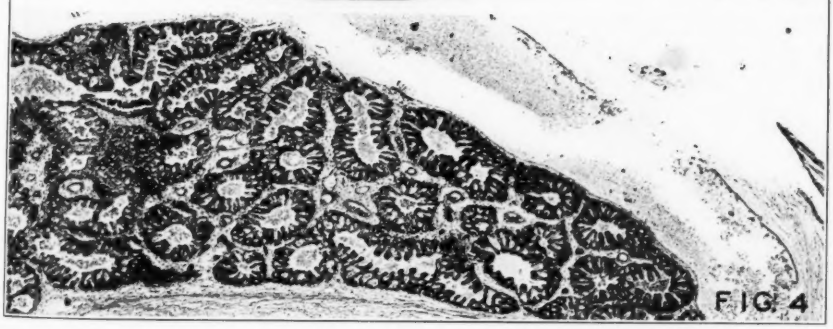


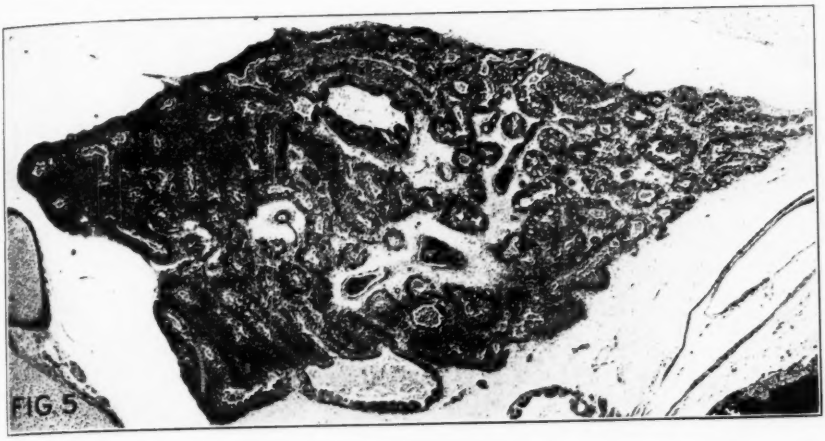
FIG. 4

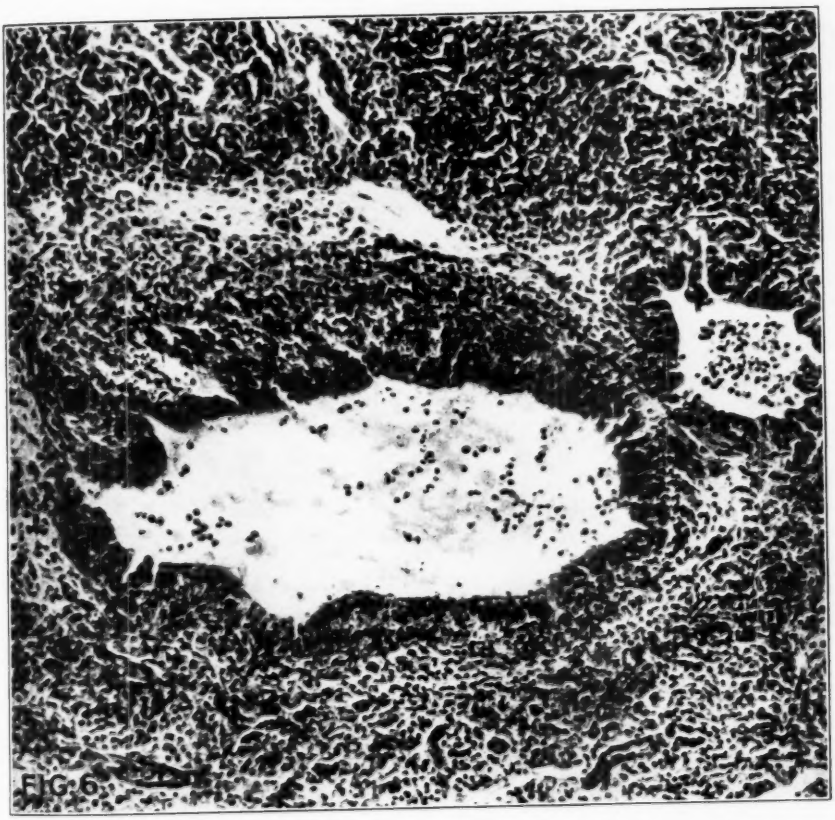
Gallavan and Goodpasture Infection of Chick Embryos with *H. pertussis*

PLATE 139

FIG. 5. Lung of embryo 6 days after inoculation of membrane with *H. pertussis*. Note intrabronchial exudate and interstitial and bronchopneumonia. Compare with Fig. 4. × 30.

FIG. 6. Higher power of bronchus in upper third of Fig. 5. Note exudate in lumen, necrosis and infiltration of epithelium, peribronchial and interstitial inflammation, and bronchopneumonia. × 190.





Gallavan and Goodpasture

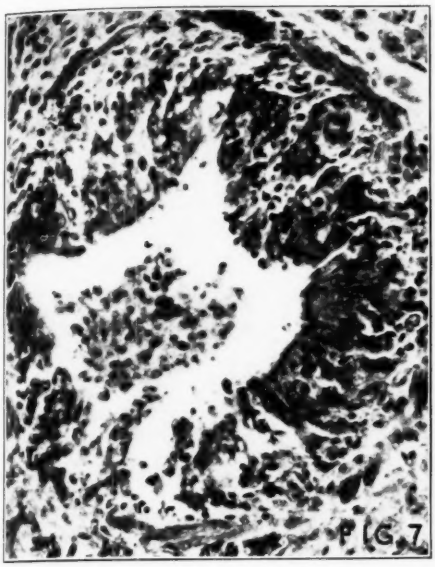
Infection of Chick Embryos with *H. pertussis*

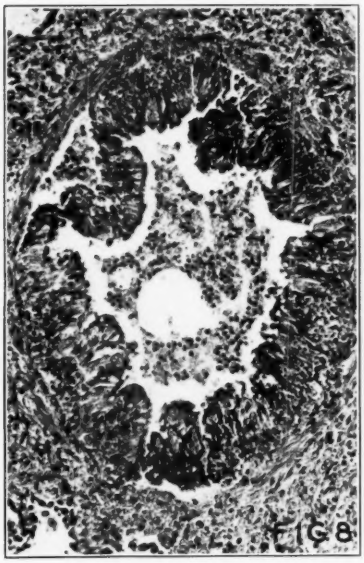
PLATE 140

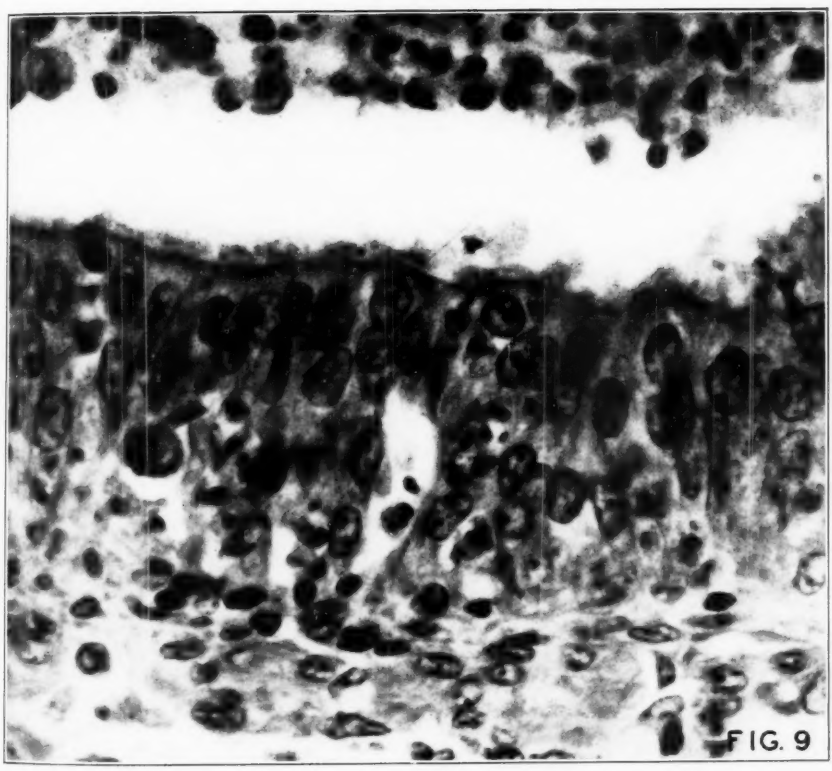
FIG. 7. Small bronchus in embryonic lung showing necrosis, desquamation and inflammation in and beneath epithelium. × 400.

FIG. 8. Small bronchus from a case of human pertussis for comparison with Fig. 7. Note similar injury and inflammation. × 120.

FIG. 9. Higher magnification of embryo bronchus showing zone of bacilli in ciliated border, and necrosis and infiltration of middle and basal layers of epithelium. Exudate in lumen. × 625.







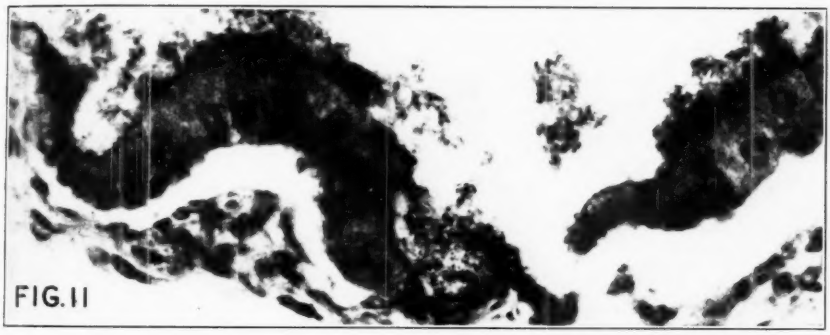
Gallavan and Goodpasture Infection of Chick Embryos with *H. pertussis*

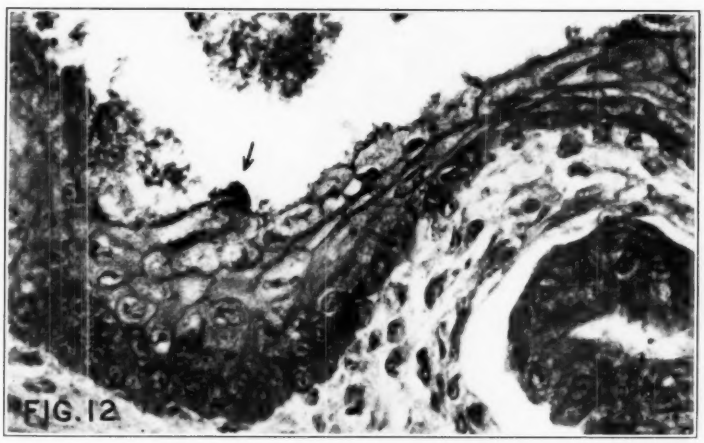
PLATE 141

FIG. 10. Bronchial epithelium of chick embryo showing bacilli among cilia and degeneration and necrosis of midzonal epithelium. × 2300.

FIG. 11. Islands of ciliated epithelium covered with masses of bacilli in expanded air sac from bronchus of chick embryo lung. × 700.

FIG. 12. Isolated ciliated epithelial cells holding clumps of bacilli in esophagus of embryo chick. × 700.





SPECIFICITY OF THE LESION OF EXPERIMENTAL MUMPS *

OSCAR BLOCH, JR., M.D.†

(From the Department of Pathology, Vanderbilt University Medical School, Nashville, Tenn.)

The object of this study was to find out whether the lesions produced in the parotid glands of *Macacus rhesus* monkeys by injecting mumps virus into Stenson's duct are microscopically specific, as stated by Johnson and Goodpasture, or non-specific and reproducible by the injection of various other substances, as Levaditi and coworkers contend.

Johnson and Goodpasture,[1] after reviewing the brief literature on the histopathology of human mumps and describing the microscopic appearance of glands taken at the height of the experimental disease in monkeys, conclude that the essential lesion produced by the mumps virus is focal degeneration and necrosis of the acinar epithelial cells, while the inflammatory cellular response is secondary thereto. They also describe pink staining, round, vacuolated inclusion bodies in the cytoplasm of degenerated and necrobiotic cells, and hazard the suggestion that these are specific in the mumps infection.

Levaditi and coworkers [2] agree that mumps is caused by a filtrable, glycerin-resistant pathogen, and can be transmitted to monkeys, but they concluded, on inoculating with several control substances (normal saliva, yeast, white of egg, horse serum, tapioca, herpes virus, and lymphogranuloma virus), that there was only a quantitative difference between the lesions produced and those caused by mumps. All types of inoculums produced a simple interstitial mononuclear inflammation without much epithelial involvement, and only by counting the number of lymphocytic foci in a microscopic field could one determine which glands were infected with mumps. Levaditi *et al.*, do not mention any foci of acinar degeneration and necrosis.

Since Johnson and Goodpasture used as controls only monkeys inoculated with normal monkey parotid suspensions in saline, it has seemed important to repeat their experiments, both with mumps virus and with several other control inoculums.

* Aided by grants from the Division of Medical Sciences, Rockefeller Foundation, and the Josiah Macy, Jr. Foundation.

† National Research Council Fellow in Pathology.

Received for publication July 10, 1937.

Materials and Methods

The parotid glands of 37 *Macacus rhesus* monkeys have been studied. Inoculations were made by syringe and cannula into the parotid ducts of etherized animals. In those inoculated with mumps virus the onset of mumps was detected by secondary enlargement of the glands to palpation and by pitting edema of the jowl, occurring generally about the 6th to 8th day after inoculation and accompanied often by a secondary rise of temperature. When these signs appeared the monkeys were etherized and exsanguinated and the parotid glands were fixed in a modified form of Schaudinn's fluid,* to be sectioned and stained by hematoxylin and eosin. No special stains were employed. In monkeys inoculated with control materials there was no secondary development of clinical mumps, but the animals were killed after intervals similar to those having mumps. The following inoculums were used:

1. Centrifuged saline suspension (20 per cent by weight) of the crushed parotid glands of monkeys at the peak of infection with experimental mumps. Two strains of virus were used: one, that of Johnson and Goodpasture, up to its 35th monkey passage; the other one recently derived from human saliva.
2. Centrifuged saline suspension of parotid glands infected with mumps boiled for 10 minutes.
3. Centrifuged saline suspensions (20 per cent) of the crushed parotid glands of normal monkeys.
4. Centrifuged saliva from children during the first 2 days of clinical mumps.
5. Berkefeld V filtrate of normal monkey saliva.
6. Berkefeld V filtrate of saliva from monkeys recently recovered from mumps.
7. A 5 per cent solution of granular mucin in saline.
8. A 7.5 per cent solution of soluble starch in saline.
9. Physiological saline solution.
10. Fresh white of egg.
11. Horse serum.
12. A 1:400 suspension of vaccinia virus from the chorioallantoic membranes of chick embryos.

* Saturated solution of mercuric chloride, 2 parts; 60 per cent alcohol, 1 part.

The size of the inoculum varied from 2 to 4 cc. in each duct.

All the above preparations were cultured and, with a few exceptions noted later, were found sterile at the time of injection.

Histological Examination

A number of the sections studied came from monkeys that had been inoculated with mixtures of virus tissue and saliva or serum, used in immunity and neutralization experiments, and which had died from 1 to 7 months after inoculation from tuberculosis, Flexner's dysentery, or from injuries by other monkeys. Their glands showed the late and ultimate effects of the mumps infection. No attempt was made, however, to study the entire pathogenesis of the infection from day to day with biopsies or serial killings. Nor was it considered practical to count the number of inflammatory foci, as was done by Levaditi and coworkers, since nearly every gland studied showed the greatest irregularity in distribution of lesions.

Thirty-seven parotid glands were studied which had been removed 6 to 8 days after injection with infectious human saliva or infectious monkey parotid by way of Stenson's duct. All showed essentially the same changes, of which the earliest was degeneration and necrosis of the epithelial cells in scattered acini, generally affecting first those at the center of the lobule, and later those farther out. Following focal necrosis the involved acini were invaded by monocytes and the entire periductal area, as well as the interacinar tissue in the vicinity, became infiltrated with lymphocytes and monocytes, occasional plasma cells, and rare polymorphonuclear neutrophiles and eosinophiles. There were edema and fibrin deposition in the intralobular and interlobular connective tissue of the inflamed areas, and occasional small recent hemorrhages. In any one section from a gland at the active stage of the infection all these changes could usually be seen, different lobules and different portions of one lobule showing acini with hydropic cells, or swollen or pyknotic nuclei, and also acini with necrotic débris which was being ingested by phagocytes to be replaced by edema and lymphocytic exudate. Neutrophilic leukocytes were absent, except in the ducts of one of the glands inoculated with unfiltered (contaminated) human saliva.

Cytoplasmic inclusion bodies of the type described by Johnson

and Goodpasture were found in 19 of the 37 mumps-inoculated glands. These bodies are pink staining, round or oval, 3 to 10 μ in diameter, often contain small vacuoles, and are usually surrounded by a narrow clear zone in the cytoplasm. They were discovered more frequently in the epithelial cells of acini showing degenerative changes, or adjacent to involved areas of the gland.

One gland removed 11 days after inoculation with mumps showed the same picture as that described above, *i.e.* necrosis, lymphocytes, inclusion bodies, edema and hemorrhage. The period of invasion in this case was longer and swelling did not appear until the 10th day.

Eighteen glands were examined from monkeys that had died or had been killed weeks or months after the initial mumps infection. In these glands the only positive finding was an occasional, usually small collection of lymphocytes in the periductal connective tissue, not involving the interacinar tissue. There was no fibrosis, necrosis, hemorrhage or edema, nor were inclusion bodies found.

One gland was studied which had been injected 6 days before with boiled mumps-infected gland suspension. It showed no pathological changes, with the exception of an occasional group of periductal lymphocytes.

One gland was removed at autopsy 11 days after injection with a suspension of normal monkey parotid gland. It showed a minimal number of periductal lymphocytes. An adjoining lymph node contained more monocytes in the medulla than are seen normally.

In one gland, removed 6 days after injection with the Berkefeld filtrate of saliva from a normal monkey, there was no change except the presence of a few periductal lymphocytes. The same was true of the other gland of the same animal, injected with a filtrate of saliva from a monkey that had had mumps a few weeks before.

One gland was examined 7 days after the injection of a 5 per cent solution of granular mucin in saline. It could be distinguished from a normal gland only by the presence of a few more periductal lymphocytes than are seen normally, and a few eosinophiles.

One gland received a 7.5 per cent solution of soluble starch in saline, and 7 days later showed only a slight increase of periductal lymphocytes, together with a change not seen in any other gland of this series. In several lobules of the section there were irregular areas in which the acini, though not necrotic, exhibited a disarrangement of the cells, which took a much lighter, pinker stain

than is seen normally. The appearance was somewhat similar to that of an early stage of Minkowski's degeneration of the pancreas.

One gland that had received physiological saline solution 6 days previously showed no pathological change whatever. Several normal (uninjected) glands were examined, and it was found that the presence of a few lymphocytes, or even of a small collection in the vicinity of the ducts should not be regarded as pathological.

One gland was injected with fresh white of egg. At autopsy 6 days later it appeared grossly normal and on section showed only a minimal amount of edema and periductal lymphocytic infiltration.

One gland, injected with horse serum 6 days before, showed slight edema, small hemorrhages, an increase in the periductal lymphocytes, and a few eosinophiles, but no other changes.

One section from a gland injected 6 days before with normal saliva from a man who had had mumps years before showed an increase of lymphocytes, both periductal and interacinar, an occasional polymorphonuclear leukocyte in the periductal tissues, and numerous cytoplasmic inclusions. There was no focal acinar necrosis of the type seen in mumps but an occasional cell or two, here and there, was necrotic. The inoculum in this case, being a centrifuged supernatant fluid and not a filtrate, was contaminated by bacteria.

One gland was examined on the 7th day after receiving 1 cc. of a dilution of vaccinia virus. It showed a large number of lymphocytes, both interacinar and periductal, marked edema, hemorrhage, and a small number of neutrophilic leukocytes, but no necrosis or inclusions. The clinical course was much more rapid and the gland was firmer to the touch than in mumps infection.

One monkey, inoculated in one parotid with mucin and in the other with saline solution, developed a suppurative infection of the mucin-injected parotid. The inoculum was cultured and found to contain *Staphylococcus aureus*. Sections of the glands showed multiple abscesses of the infected gland, with some fibroblastic reaction and many plasma cells. Edema, hemorrhage, and lymphocytic infiltration were also abundant, and there were large and small areas of necrosis, some of which could not be distinguished from those caused by mumps.

The saline-injected gland showed no suppuration, but edema, heavy lymphocytic infiltration, acinar necrosis and inclusion

bodies were found. This lesion was indistinguishable from that of mumps and an adequate explanation of it is not at hand as it was not reinoculated into the monkeys to rule out mumps. However, there was no gross edema of the gland, which was of normal size.

Two other glands examined showed staphylococcal infections: one, which also showed mumps, from a contaminated inoculum; the other, 7 months after inoculation, from a traumatic infection. Both showed suppurative foci with polymorphonuclear leukocytes in marked predominance.

Conclusions

From the above observations it is concluded that:

1. Focal acinar necrosis, *i.e.* necrosis involving all or most of the cells of an acinus, is the fundamental lesion of experimental mumps, the lymphocytic reaction, edema and hemorrhage being subsequent factors.

2. None of the inert or infectious substances injected give this particular type of necrosis, though some of them may cause the death of a cell here and there or produce an abscess destroying large numbers of acini.

3. Cytoplasmic inclusion bodies, though found in over half the cases of mumps, may also be found in glands injected with normal saliva, and cannot therefore be regarded as a specific sign of mumps infection.

4. The abundance and interacinar location of the lymphocytic exudate can be suggestive evidence that the lesion is due to infection rather than to an inert substance, but do not aid in distinguishing between different types of infection.

It should be stated that in no instance following injections of the parotid with material other than that of mumps did a secondary enlargement, with edema of the gland associated with elevation of temperature, occur. This seems to be characteristic of mumps infection, as is likewise the histopathological lesion.

REFERENCES

1. Johnson, C. D., and Goodpasture, E. W. The histopathology of experimental mumps in the monkey, *Macacus rhesus*. *Am. J. Path.*, 1936, **12**, 495–510.

2. Levaditi, C., Martin R., Bonnefoi, A., and Schoen, R. L'étiologie des oreillons. *Compt. rend. Acad. d. sc.*, 1935, **201**, 174–176.

CHANGES IN THE TEETH FOLLOWING PARATHYROIDECTOMY *

I. The Effects of Different Periods of Survival, Fasting, and Repeated Pregnancies and Lactations on the Incisor of the Rat

I. Schour, D.D.S., Ph.D., S. B. Chandler, M.D., and W. R. Tweedy, Ph.D.†

(From the Department of Histology, University of Illinois College of Dentistry, and the Departments of Anatomy and Biochemistry, Loyola University School of Medicine, Chicago, Ill.)

Introduction

Since the incisor teeth of the albino rat reflect with a high degree of accuracy and sensitivity the fluctuations in calcium and phosphorus metabolism, they have proved to be a valuable indicator in studying the physiology of the parathyroid glands.

The purpose of this investigation was, therefore, to study the response of the incisor of the rat to the following experimental conditions: (1) short survival following parathyroidectomy (3–20 days); (2) long survival following parathyroidectomy (40 days or more); (3) periodic fasting superposed on parathyroidectomy of long survival; and (4) repeated pregnancies and lactations superposed on parathyroidectomy of long survival.

Review of Literature

Erdheim [1] was the first to study the dental changes in the incisors of parathyroidectomized rats. He reported the following changes. The enamel surface showed opaque spots and the teeth fractured 6 to 10 weeks after the operation. The dentin showed incomplete or no calcification, a wide predentin, and vascular inclusions. The enamel epithelium was atrophied and folded. Atypical enamel formation occurred within the enamel epithelium.

Toyofuku [2] made a more detailed histological analysis of the incisors of 27 rats that survived 1–135 days after parathyroidectomy. He confirmed Erdheim's findings and added observations made on roentgenograms. The findings of Erdheim and Toyofuku have been repeatedly confirmed and supported by a number of

* This investigation was aided by a grant to one of us (I. S.) from the Committee on Scientific Research of the American Medical Association and from the Graduate School Research Board of the University of Illinois.

† Mr. M. Engel rendered valuable assistance in several phases of this work.

Received for publication July 15, 1937.

investigators (Preiswerk-Maggi,[3] von Spreter[4]). In the above investigations diet was an important determinant but unfortunately the exact diets used were not recorded.

In 1911 Erdheim[5] removed the parathyroids and transplanted them in the abdominal wall of the same animal. He thus produced a temporary state of parathyroidectomy which was registered in the form of an imperfect calcification of the dentin that was laid

TABLE I

Data on 18 Parathyroidectomized Animals of Group I Arranged According to Postoperative Survival

Rat. No.	Age at operation	Weight at operation	Postoperative survival
	days	*gm.*	*days*
110	46	84	3
82	21	35	4
88	79	138	5
36	23	47	6
50	24	30	7
55	24	31	8
9	32	52	10
53	24	28	14
54	24	30	13
43	21	37	13
44	21	37	14
52	24	27	14
51	24	25	15
41	21	35	15
42	21	38	15
49	23	31	16
37	23	53	20
38	23	53	20

down during the time that elapsed between the removal of the parathyroids and the "taking" of the transplant. This zone Erdheim called the transplantation stripe. In 1914, in his classic work "Rickets and Parathyroids," Erdheim[6] pointed out that changes in the incisors of rachitic rats were the same as those in parathyroprivic rats.

MATERIAL AND METHODS

This report is based on 100 parathyroidectomized albino rats. In the great majority the age at the time of operation ranged from 21 to 79 days (Table I). The range of postoperative survival was from 3 to 159 days (Tables I to III).

TABLE II

Data on 57 Parathyroidectomized Animals of Group II Arranged According to Postoperative Survival

Rat No.	Age at operation	Weight at operation	Postoperative survival *
	days	*gm.*	*days*
67	55	106	39
68	55		39
25A	27	57	42
27A	27	47	43
14	117	158	43
45	21	36	47
47	21	37	47
48	21	34	47
6B			48
7B			48
9B			48
10B			48
3B			49
5B			49
61	111	152	52
6A	40	103	56
10A	41	76	56
13A	41	68	56
14A	41	68	56
4A	39	84	57
5A	39	70	57
17A	42	69	57
1A	38	62	58
3A	38	54	58
1	32	74	66
2	32	76	66
3	32	86	66
4	31	74	66
7	32	77	66
31	39		82
901	34		83
903	34		83
904	34		83
905	34		83
906	34		83
907	34		83
29	23	49	98
30	23		98
32	23	49	98
113	47	110	120
116	47	123	120
119	23	32	125
120	23	33	125

* These animals were treated with calciferol or parathyroid extract several days before sacrificing. The indicated postoperative survival refers to the survival up to the time of treatment.

TABLE II (*Continued*)

Rat No.	Age at operation	Weight at operation	Postoperative survival *
	days	*gm.*	*days*
123	23	34	130
125	23	34	133
126	23	29	133
108	46	99	135
111	46	83	135
112	46	97	135
100	41	92	140
101	41	107	140
96	46	103	157
64	93	146	157
66	55	108	157
91	82	192	159
92	82	168	159
93	82	169	159

* These animals were treated with calciferol or parathyroid extract several days before sacrificing. The indicated postoperative survival refers to the survival up to the time of treatment.

TABLE III

Data on 14 Parathyroidectomized Animals of Group III Subjected to Fasting Every 7th Day

Rat No.	Age at operation	Weight at operation	Postoperative survival
	days	*gm.*	*days*
H 40	63	137	105
H 42	63	122	105
H 44	65	110	105
H 50	67	147	105
H 52	67	159	105
H 53	67	162	105
H 55	67	185	105
H 56	67	160	105
H 70			105
H 80	70	87	105
H 86	70	69	105
H 87	71	117	105
H 90	72	95	105
H 92	72	101	105

On the basis of the experimental history, these animals may be divided into 4 groups:

Group I consists of 18 animals of short postoperative survival (3–20 days, Table I).

Group II consists of 57 animals of longer postoperative survival (39–159 days). These animals were treated with calciferol or parathyroid extract several days before death (Table II).* They were included in this study because the histological findings in that portion of the incisor formed prior to treatment were fully representative of the changes produced by parathyroidectomy alone. The histological effects of the treatment on parathyroprivic dentin which was found to be confined to the portion that was formed and calcified during and subsequent to the time of treatment are described in the subsequent report.[7]

Group III consists of 14 animals of a postoperative survival of 105 days. During this period the rats were subjected to fasting on every 7th day (Table III).

Group IV consists of 11 rats of long postoperative survival (4 months to 1 year) which were subjected to repeated pregnancies and lactations. These animals are part of the series described in a previous report by one of us (Chandler [8]).

Similar histological studies were carried out on 27 litter-mate controls, of which 6 were subjected to unilateral parathyroidectomy; 6 were subjected to a sham operation in which the thyroids and parathyroids were exposed; and 9 were treated with parathyroid extract or calciferol. In addition, the histological data of a number of controls from the same colony and within the same age limits as the experimental animals were available from a previous study (Schour, Tweedy and McJunkin [9]).

The controls showed a normal histological picture in the dental tissues. The 9 animals that were treated with parathyroid extract or calciferol showed a normal picture in the portion of the incisors that was formed and calcified previous to the treatment.

The diet consisted of Purina Fox Chow *ad libitum* which has been found to be adequate in calcium, phosphorus, proteins, carbohydrates and fat. In addition the animals were given cheese and

* We are indebted to Mead Johnson and Company for the calciferol used in these experiments. A portion of the parathyroid extract was supplied through the courtesy of Eli Lilly and Company.

cabbage twice and beef once a week. The animals belonged to the colony of the Loyola University Medical School and were originally derived from the Wistar strain.

Parathyroidectomy was performed by one of us (S. B. C.). Serial sections were prepared of all tissue removed and all incompletely parathyroidectomized animals were discarded. While the incidence of accessory parathyroid tissue was not determined by histological examination of serial sections of the entire neck regions from all animals used, similar studies recently carried out on 68 animals from the same colony revealed 5 animals with accessory tissue. We therefore regard the rats as completely parathyroidectomized since the amount of accessory tissue observed in the animals of this colony is quite small in every instance studied and the number of animals possessing residual parathyroid is less than 10 per cent.

After parathyroidectomy the rate of eruption of the incisors was measured. The heads of the animals were fixed in 5 per cent formalin immediately after death. They were then cut midsagittally and X-rayed. The tissues were washed, decalcified in 5 per cent nitric acid, dehydrated, and embedded in celloidin. Sections were cut in serial order and stained with hematoxylin and eosin. The majority were longitudinal sagittal sections of the upper and lower incisors. A small number of ground sections were prepared.

Gross Findings

Findings in the Living Animals

Gross Changes: The gross changes were similar to those repeatedly reported in the literature but appeared to occur later in respect to survival time. No gross changes were observed in Group I. In Group II the exposed enamel surface occasionally showed unpigmented spots and presented an opaque appearance. These changes were found to be more consistent in Group III. The defective pigmentation and opaque appearance of the incisors were more marked in Group IV. In most instances the teeth were elongated.

The exposed portion of the enamel frequently showed a fine but distinct ring-like stratification which could be recognized with the naked eye.

Measurements of Rate of Eruption: The weekly rate of eruption of the upper and lower incisors in 10 parathyroidectomized animals was determined for an average period of 10 weeks. These rats were obtained from Group II and were measured previous to their treatment with parathyroid extract or calciferol. The results indicate an average normal weekly rate of 2 mm. in the upper and 2.8 mm. in the lower incisors. Similar measurements on normal animals were in the same range.

Radiographical Findings

The roentgenogram of the incisor of the normal mature rat presents a curved and partially hollow cylinder with a sharp incisal edge. The shadow of the tooth has a smooth and sharply delineated external outline and is dense in its distal third or half. Beginning with the middle third, because of the increasingly large radiolucent pulp, this shadow splits into a tapering convex (labial) and a tapering concave (lingual) portion (Fig. 3).

Group I. No radiographical changes were evident in this group.

Group II. About 30 per cent of the animals in this group showed evidence of disturbances. Formation disturbances were recognized on the basis of the irregular contour of the enamel surface. Disturbances in calcification were indicated by radiolucent areas in the lingual dentin of some of the lower incisors (Figs. 4–6). These areas were incremental in position. Corresponding areas were found to take an eosin stain in decalcified histological sections (Pl. 144, Figs. 1, 2) and showed prominent interglobular dentin in ground sections (Figs. 11, 12). The same zones were radiolucent in the Grenz X-ray plates. The latter permit microscopic examination of roentgenograms of ground sections and were kindly prepared for us by Dr. E. Applebaum of the Department of Oral Histology of the Columbia University Dental School. Three animals (5 per cent) showed fractures.

Group III. The changes in this group were similar to those in Group II but occurred in 50 per cent of the animals. Two animals (14 per cent) showed fractures.

Group IV. Each of the animals of this group showed pathological alterations. While the roentgenograms show very definite differences in the density of calcification they also indicate disturbances in formation. The irregular contour of the enamel surface,

the distortion of the incisors and the irregular width of the labial alveolar periosteum are seen in the majority of the animals (Figs. 7–10).

Each animal showed incremental radiolucent zones in the lingual dentin of the lower incisors. Many showed similar zones in the upper incisor and a few showed them in the enamel as well. The pulpal outline is as a rule very distinct and extends to the incisal edge. The prominent width of the pulp in the anterior portion of the incisor indicates an absence of deposition or a severe deficiency in the calcification of the dentin. Six animals (55 per cent) showed fractures in the incisors.

Elongation of teeth, extra- or intra-alveolar fractures, and blunted incisal bevels were found more frequently in this series than in the preceding groups (Figs. 7–10).

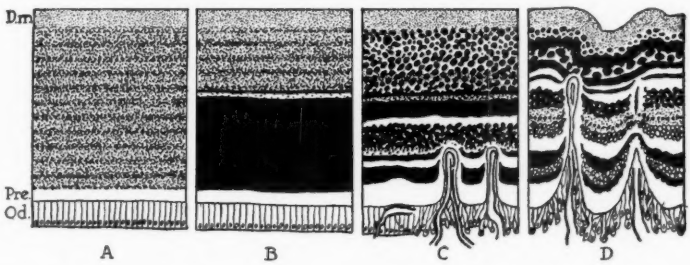
Two animals in this group show an exceptionally severe enamel hypoplasia in the proximal zone (Fig. 9). On first glance they remind one of the characteristic changes seen in long survival following hypophysectomy (Schour and van Dyke [10]). However, the changes differ from those seen in hypophysectomy in respect to the presence of a number of radiolucent incremental zones, and of elongation and the absence of the obliteration of the pulp.

Histophysiology of the Incisor of the Rat

Since the incisors of the albino rat reflect with a high degree of accuracy and sensitivity the fluctuations in calcium and phosphorus metabolism, they have proved to be valuable indicators in studying the physiology of the parathyroid glands.

For a statement of the histophysiology and growth pattern of this tooth, the reader is referred to a brief report by Schour and Steadman.[11] In our present problem we are concerned primarily with the calcification pattern of the dentin. This tissue consists of a collagenous matrix in which lime salts are embedded. Both the apposition and the calcification of dentin proceed along a rhythmic incremental pattern at the rate of 16 μ per 24 hours (Schour and Hoffman [12, 13]). There is a small time difference in which formation precedes calcification. As the dentin-forming cells recede pulpally and migrate distally, they continually form new dentin matrix, while the matrix formed on approximately the previous day is calcifying.

The matrix is calcified in the form of globules which follow the typical Liesegang ring formation; when one globule enters the sphere of influence of another a fusion occurs. Normally calcified dentin appears homogeneous because of complete fusion of a large number of spherical areas. But even in the normal condition, the successive layers of the dentin are not equally well calcified. Well calcified layers which stain more readily with hematoxylin alternate regularly and rhythmically with less well calcified layers which stain more readily with eosin. Thus there arises in the dentin a stratification which consists of pairs of dark and light increments (Text-Fig. 1, A). These pairs are of uniform width



Text-Figure 1. Schematic representation of the state of calcification of dentin of the incisor of the normal rat (A), and parathyroidectomized rat (B, C, and D).

A. Stipling represents normal dentin and shows the daily calcification and growth rhythm. The predentin (Pre) next to the normal odontoblasts (Od.) is even in width and white. The mantle dentin (D. m.) is located next to the enamel and is shown in fine dense stippling.

B. The stippled zone represents the preoperative dentin; the dark line followed by the narrow dotted zone represents the hematoxylin staining line and eosin zone in the dentin that is being formed and calcified at the time of and shortly after the operation. The black zone extending to the predentin is the heavy hematoxylin staining dentin found in the early parathyroprivic condition. B is representative of Group I.

C. The irregularly staining stratification zones in the parathyroprivic dentin of long survival are represented by the different size and spacing of globules (black). Note the greater width of the predentin and also the odontoblastic changes associated with the vascular inclusions. The dentin immediately adjacent to the inclusions like the predentin is white. C is representative of Group II.

D. The dentin surfaces bordering the enamel and the pulp are distorted. The incremental stratification zones are more irregular and show an arcade-like arrangement near the vascular inclusions. Odontoblastic changes are more prominent. D is representative of changes in Groups III and IV.

(approximately 16 μ) and give the dentin its regular and characteristic incremental calcification pattern (Fig. 15, and Text-Fig. 1, A).

The latter presents different aspects in the various portions of the same tooth. There is an anteroposterior calcification gradient which expresses itself in the fact that the incremental pattern is most distinct in the distal portion of the incisor and difficult to recognize in the proximal portion.

There is also a pulpo-mantle calcification gradient which runs parallel with the course of the dentinal tubules from the pulpal surface to the mantle dentin. The dentin immediately next to the pulp consists of the newly formed matrix which is not yet calcified and is called the predentin. The latter is divided into early and late stages which stain differentially with hematoxylin and eosin. The transitional zone between dentin and predentin is known as the intermediate dentin which stains both with hematoxylin and eosin (Schour and Rogoff [14]). It is apparently in this intermediate zone that the dentin reaches its stage of mature calcification.

The normal process of secondary calcification which runs along the course of the dentinal tubules results in an increased calcium deposition which is in direct proportion to the age of the particular dentin increment, or its distance from the pulp. The dentin farthest away from the pulp is called the mantle dentin and appears to be most calcified.

When the globular calcification is incomplete, the dentin areas which are between the globules take an eosin stain and constitute interglobular dentin.

Occasionally a normal control will show an incremental layer which is conspicuous because of its preferential staining reaction with eosin. Such a reaction presents itself in the extremely sensitive dentin without any apparent outward manifestation in the habitus of the animal. This is due to the fact that calcification of the dentin is extremely sensitive to disturbances in calcium metabolism.

Histopathology of the Incisor of the Parathyroidectomized Rat

Parathyroidectomy results in disturbances of calcification and growth. The essential character of the reaction varies with the

TABLE IV

Dental Changes in Parathyroidectomized Albino Rats Arranged According to Experimental Groups I–IV

Group	Experimental history		Gross changes	Fractures of incisors	Radiographic evidence of disturbances in calcification and formation	Histological changes			
						Disturbances in calcification		Disturbances in formation	
	Number of rats	Survival period				Enamel	Dentin	Enamel	Dentin
		days							
I (Short survival)	18	3–20	None	None	None	None	1. Sharp hematoxylin staining stripe in the dentin calcified about the time of the operation. 2. Postoperative dentin frequently stains deeply with hematoxylin	None	None
II (Long survival)	57	39–181	Opaque enamel. White spots in enamel	In 5% of rats	In 30% of rats	1. Persistence of enamel matrix in distal portion. 2. Accentuated incremental line in matrix	1. Postoperative dentin interglobular. 2. Irregular and accentuated incremental stratification and zoning. 3. Vascular inclusions. 4. Calcospherites in distal pulp. 5. Predentin slightly wider than normal	Enamel epithelium disorganized in distal half. Occasionally isolated in connective tissue. Ameloblasts shortened or displaced by connective tissue	1. Vascular inclusions. 2. Irregular dentinopulpal and dentinoenamel border. 3. Odontoblasts crowded and disorganized near vascular inclusions
III (Fasting every 7th day)	14	105	As in Group II, but more consistent	In 14% of rats	In 50% of rats	More marked than in Group II. Enamel matrix stratified	More severe than in Group II	More severe than in Group II. Prominent atrophy of enamel epithelium. Enamel surface distorted	More severe than in Group II
IV (Repeated pregnancy and lactation)	11	120–360 (approximately)	As in Group III, but more marked; teeth elongated	In 55% of rats	In 100% of rats	More marked than in Group III	More severe than in Group III	More severe than in Group III	2/3 normal width of dentin

postoperative survival. From the histological point of view, our material may be classified according to the same grouping that was obtained on the basis of the experimental history (Table IV, Text-Fig. 1).

Group I. Changes During Brief Survival (3 to 20 Days)

In this group the reaction to parathyroidectomy is an increase rather than a decrease in dentin calcification. The typical effect is well illustrated in Rat 55 which lived 8 days after the operation (Text-Fig. 1, B, and Figs. 1, 2). The postoperative dentin stains more deeply with hematoxylin than the preoperative dentin. At the border between the two there is a fine sharp line which stains deeply with hematoxylin and may be followed especially on the lingual portion by a narrow zone of eosin staining dentin (Fig. 2).

It has been experimentally demonstrated [13] that the rate of deposition of dentin in the rat incisor is approximately 16 μ in 24 hours. This constant was used in our histological analysis. Measurements in the incisor of this animal and others of Group I indicate that the first hematoxylin stripe corresponds closely with the time of the operation.

In some animals of this group only the dentin of about the first 15 days after parathyroidectomy shows the deeper staining reaction, while the dentin laid down in the subsequent period shows a staining reaction which is normal and similar to that of the preoperative dentin.

Group II. Changes During Longer Survival (More than About 35 Days)

In this group the reaction to parathyroidectomy is one of progressive deficiency in the calcification and formation of dentin and enamel (Text-Fig. 1, C). The findings in Rat 29 are given in greater detail below and are illustrative of this group. Since replacement therapy with parathyroid extract was not initiated until 98 days after parathyroidectomy (Table II), we may consider this animal as parathyroprivic for 98 days. Four days following treatment the animal was sacrificed. A histopathological description of the upper left incisor is presented.

Disturbances in Calcification:

Labial Dentin: The disturbances in the calcification pattern of the dentin differ in intensity in different portions of the incisor. The proximal third of the labial dentin is characterized by considerable dispersion of interglobular dentin. The predentin is wider than normal and its border against the calcified dentin is invaginated by vascular inclusions. In the distal third the globular dispersion is masked by an accentuated stratification pattern which consists of an irregular alternation of deep hematoxylin staining areas and eosinophilic interglobular areas.

Vascular Inclusions: Vascular inclusions make deep penetrations, disturb the normal pattern, and often extend throughout the entire width of the dentin except for the portion next to the enamel (the mantle dentin). The incremental layers curve sharply and tend to follow the direction of the inclusions. The interruptions thus give an arcade-like appearance to the calcification pattern (Fig. 14) and are especially distinct because the dentin immediately adjacent to them is not calcified and stains like predentin. Concentric calcospherites are found in the most distal portions of the pulp (Fig. 16).

Lingual Dentin: In the lingual dentin the contrasting strata are especially outstanding, particularly in the distal third of the incisor. Here we have incremental eosin staining areas, some of which are as wide as 150 μ alternating with narrower zones which stain deeply with hematoxylin. The position of the wide eosin staining areas corresponds with that of the incremental radiolucent zones seen in the roentgenograms of the entire teeth and in the Grenz-ray pictures of the ground section of the incisors of parathyroidectomized rats of similar history. The entire dentin effect is one of irregular zoning of different densities in calcification superposed on the normal rhythmic stratification (Figs. 13, 14, 18–20).

Fractures: Three animals in this group show intra-alveolar fractures (Figs. 14, 17) which are as a rule transverse to the long axis of the incisor. A fracture which runs along the incremental pattern is shown in Figure 17. Incisal stress apparently split the tooth along an incremental zone which was poorly calcified and which was bordered by better calcified incremental layers on either side.

Enamel: The enamel space contains a remnant of the organic matrix at the level of the alveolar crest. The proximal third shows a sharply accentuated incremental line in the matrix. The position of this line indicates that it was produced approximately 2 weeks previous to death.

Disturbances in Formation:

Dentin: The formation of the dentin is interrupted by vascular inclusions and indentations, especially in its labial portion. The dentino-pulpal border is accordingly irregular (Plate 144, Fig. 1 [7]). The odontoblasts are disorganized, especially in the distal third. Here they are more crowded and their long axis is not parallel with the direction of the dentinal tubules.

Enamel: The enamel epithelium is disorganized in the distal half. The epithelial projections are elongated, lose their parallel arrangement and are farther apart. The ganoblasts are shortened and together with the remaining epithelium are in some places displaced by intra-epithelial cyst-like formations. Small isolated isles of epithelium are found in the labial alveolar periosteum.

Near the alveolar crest the enamel epithelium is completely absent and replaced by connective tissue which is filled in part with an eosin staining fluid. At this point the organic enamel matrix is still present. The labial alveolar periosteum is wider than normal.

Group III. Changes in Animals that Survived 105 Days and were Subjected to Fasting on Every 7th Day

The findings in Rat 50 are illustrative of this group (Text-Fig. 1, D). This animal was permitted to live for 105 days following operation. There was a deviation from the usual feeding routine in so far as the animal was submitted to fasting every 7th day. The histopathological changes of the upper left incisor are described below.

Disturbances in Calcification:

Dentin: The changes are more severe than those observed in Group II. The stratification is markedly accentuated (Fig. 13). The dentin is interrupted by undercalcified areas surrounding vascular inclusions. The predentin is poorly calcified and its width in the proximal labial third is 40 μ.

Calcospherites similar to those seen in Figure 16 were found in the most distal portions of the pulp of the lower incisors. Their presence in the upper incisors could not be determined because the sections did not pass through the midsagittal plane in the distal portions.

Enamel: Disturbances in the enamel calcification are associated with atrophic changes in the enamel epithelium. Proximally the outer enamel surface is distorted and the enamel matrix is stratified (Fig. 12). A portion of the enamel matrix persists as far as the gingival crest. Here, too, the surface is distorted (Fig. 13).

Disturbances in Formation:

Dentin: In the distal region the dentin surface shows distortions at levels that also show corresponding disturbances in the enamel. Formation is interrupted by vascular indentations and inclusions. The changes in the odontoblasts are similar to those seen in Rat 29.

Enamel: The enamel epithelium exhibits a series of severe atrophic changes. Proximally some of the cells have completely degenerated and enamel globules are found in the cellular débris. Here the enamel matrix is, for the most part, replaced by an accumulation of eosin staining fluid. In the middle third the epithelium is folded and has retrogressed to a low cuboidal type. Occasionally masses of closely packed atrophic enamel epithelium protrude into the enamel space or extend into the labial alveolar periosteum. The enamel at these points is undercalcified, as evidenced by the persistence of enamel matrix as seen in Plate 144, Figure 4.[7] This reaction repeats itself distally (Fig. 13).

In this group the alveolar and jaw bones show an intense staining of the cementing lines and the lining of the Haversian canals with hematoxylin. A similar reaction was also observed in Group II.

Group IV. Changes in Animals that Survived for a Period of 4 Months to 1 Year and were Subjected to Repeated Pregnancies and Lactations

The histological changes in this group confirm the severe disturbances seen in the roentgenograms (Figs. 7–10). The alterations are much more severe and intense than in those of Group III (Text-Fig. 1, D).

Disturbances in Calcification and Formation:

The calcification disturbances in the dentin differ from those described in Groups II and III in respect to their increased intensity but are similar in type. The enamel shows prominent and persistent incremental stratification (Fig. 12). The organic matrix is found to persist in the majority of animals throughout the extent of the enamel space and adheres to the exposed portion of the incisors.

The disturbances in formation are relatively more prominent than the increased disturbances in calcification. The enamel surface and the dentino-enamel and dentino-cemental borders are extremely wavy or distorted. In 2 animals there is a severe buckling up of the enamel and the dentin in the proximal portion (Fig. 9) which reminds one of similar distortions seen characteristically in hypophysectomy.

In a number of animals the dentin at the distal end is half or two-thirds the normal width. Ankylosis of the labio-alveolar periosteum with the dentin is found in 1 animal. Calcospherites in the pulp are seen more frequently in this group than in the previous groups. The enamel epithelium frequently shows early premature atrophy.

Discussion

Comparison of Our Findings with Those Reported in the Literature

Our findings on the dental changes in the incisor of the parathyroprivic rat differ from those reported in the literature particularly by Erdheim [1] and Toyofuku [2] in the following respects:

1. The rate of eruption measured in 10 rats was normal. Gottlieb [15] reported retarded eruption.

2. In early survival periods (up to about 20 days) the parathyroprivic dentin shows denser calcification than the preoperative dentin. Previous investigators reported an immediate impoverishment or absence in calcification (Erdheim,[5] Toyofuku,[2] von Spreter [16]).

3. For any given similar period of survival longer than about 20 days our findings show disturbances of a type similar to that reported by previous investigators but of lesser severity.

4. Our findings show less prominently the rachitic-like changes

that are emphasized in the literature in respect to the presence of wide predentin and a transitional interglobular dentin zone (Toyofuku,[2] Erdheim [6]).

The Possible Basis for the Difference in Results

In evaluating the possible reasons for the difference in results in the various investigations the following factors should be considered: completeness of operation, stock of animals, age at operation and diet.

It is justifiable to assume that our surgical removal was as complete as was the case in previous investigations. Our parathyroidectomy was as complete as is experimentally possible. The histological analysis of all the tissue removed at the time of the operation and the blood calcium were used as an index of the completeness of the removal. Histological studies showed the presence of accessory tissue in less than 10 per cent. These results confirm earlier findings (Hoskins and Chandler [17]) and indicate that accessory parathyroids do not vitiate results in a large and well controlled series of experiments.

Our animals were of the Wistar strain, which has become a standard for experimental purposes, but this is not the probable reason for the differences in results. The age at operation is not a significant factor in the difference in effects because our experimental series included animals of the same ages used by previous investigators; moreover, most of the animals were operated on during the period of most marked susceptibility to parathyroidectomy (Hoskins [18]).

The differences can be explained largely on the basis of dietary factors. Our basal diet was adequate in calcium (1.41 per cent) and phosphorus (0.98 per cent) and in vitamin D. The diet employed by Erdheim and Toyofuku was very likely deficient in vitamin D and calcium. Shelling [19] points out that the dietary knowledge was incomplete at the time of Erdheim's experiment and that the diet of Erdheim's animals consisted chiefly of bread.

Erdheim [6] concluded in his work on rickets and parathyroids that the dental changes in rickets were similar to those in parathyroidectomy. This conclusion is not supported by our findings. It is likely that the basal diet of Erdheim's parathyroidectomized rats was at least slightly rachitogenic. Shelling [20] demonstrated

the varying effects of changes in diet in their effect in fracture healing in parathyroidectomized animals. Shelling's findings differed from those of Erdheim apparently because of a difference in the diets that they employed.

Von Korenchevsky [21] states that the macroscopic changes in the teeth of his parathyroprivic animals were not as severe as those observed by Erdheim. Von Korenchevsky found the most profound changes in animals that had a deficiency or lack of fat soluble vitamin A. In 1922 he did not realize that on the basis of his diet this also meant vitamin D deficiency. He was unable to confirm Erdheim on the rickets-producing effect of the removal of the parathyroid. He points out that "diet is an all important factor which can by itself produce profound changes in the skeleton and comparatively little, if any, attention was paid to the diet by the majority of the above investigators," (referring to Erdheim and others).

Von Spreter [16] does not give the diet employed in his work published in 1936. In one of his reports [22] dealing in part with parathyroidectomy, he states that the diet consisted of grain plus various breads soaked in water. It appears that the findings of previous investigators were a result of at least slight nutritional deficiencies superposed on parathyroidectomy.

It is possible that slight deficiencies in the diet are insufficient to produce noticeable effects in normal animals but may contribute to more marked effects when combined with parathyroidectomy.

The available data indicate clearly that the dietary effects exert significant influence on the course of events in the parathyroprivic animal.

The Importance of the Survival Factor: Possible Basis for the Denser Calcification of the Parathyroprivic Dentin Formed During Short Survival Periods

The findings show that a correlation exists between the duration of the survival period and the histological response. Thus, in our group of animals surviving from 3 to 20 days the evidence indicates an increase in the density of dentin. Survival beyond this period is associated with a progressive impoverishment in dentin calcification and a progressively accentuated alternation of over- and under-calcification.

The data available in the literature indicate a retention of calcium in the body particularly in early survival periods of parathyroidectomy. The occurrence of hypocalcemia is well established. In a complete analysis of calcium and phosphorus metabolism in the parathyroidectomized rat, Bülbring [23] reports an increased calcium and phosphorus retention in early parathyroidectomy which lessens as the survival period progresses. The fact that hypercalcification of dentin occurs in the early survival periods after parathyroidectomy would seem to be in agreement with this observation. Bülbring, however, points out that the retained calcium is deposited in the soft tissue rather than in the bone. Our evidence seems to indicate that given a diet that is adequate in calcium, phosphorus and vitamin D, there is retention also in dentin. This harmonizes with Erdheim's calcio-protective law, according to which the growing dentin is a tissue which is given special protective preference in cases of disturbances in calcium metabolism. Furthermore, calcified dentin, unlike bone, is not subject to calcium withdrawal (Albright, Aub and Bauer [24]).

The lessened retention of calcium and phosphorus in later periods and the establishment of a relatively more stationary level of lowered blood calcium may account for the characteristic and better known calcification deficiencies in dentin during longer periods of survival.

The Effect of Fasting

Fasting on every 7th day resulted in an increase in the severity of the histopathological changes. The most prominent effect was an accentuation in the stratification as shown in Figure 13.

Measurements of the intervals between the prominent eosin staining stripes did not correspond closely with the intervals between the fasting periods, as might have been expected. It appears that the rhythmic factors that make for the characteristic accentuated fluctuation of calcification in the dentin following parathyroidectomy are more basic and are not promptly, if at all, modified by short fasting periods.

It is probable that in our experiment fasting exerted its influence primarily through the resulting decrease in the intake of calcium and other food factors that promote calcification.

Effects of Repeated Pregnancies and Lactations

The most severe histopathological alterations were found in the series of rats (Group IV) that were subjected to repeated pregnancies and lactations. The greater drain on calcium is probably chiefly responsible for the more severe reaction. Our findings do not support the view that the greater demand for calcium resulted in a withdrawal of calcium from the teeth. It is more likely that the calcium needed by the normal growing increments of enamel and dentin was much less available under the repeated pregnancies and lactations than under conditions of parathyroidectomy alone.

If calcium withdrawal were responsible for the disturbed calcification in the incisors, a similar disturbance should be recognizable in the molars. This was not the case. The latter showed normal calcification because they had completed their formation and calcification before or soon after parathyroidectomy and subsequent modifications did not occur.

It is also possible that the combined effect of parathyroidectomy and repeated pregnancies and lactations results in a disturbance of the interrelations of the endocrines and thereby aggravates the changes that are characteristic of parathyroidectomy alone.

The greater incidence of fractures in this group can be correlated with the more severe fluctuations in the calcification pattern of the dentin. It appears that the extreme fluctuations of excessive calcification and lack of calcification, rather than the lack of calcification alone, are responsible for the readiness of the parathyroprivic dentin to fracture.

Possible Factors in the Causation of Developmental Changes

Developmental changes in the incisor of the parathyroprivic rat as a result of long survival periods were found chiefly in the enamel organ and also involved to a lesser extent the odontoblasts. These cellular injuries, as well as the vascular inclusions, tend to be localized and are associated with the local disturbances and interruptions of the calcification pattern that is characteristic for the parathyroprivic condition.

The fact that the dentin showed a very narrow width in a number of the rats of Group IV suggests that the life span of the odontoblasts was shortened considerably in those cases.

Variations in the calcium and phosphorus content of the in-

ternal medium of the cells over a prolonged period may very likely account for the atrophic changes. There is also the possibility that the absence of the parathyroid hormone over a long interval might exert a direct effect on the cell metabolism or on growth. The ganoblasts seem to be especially affected.

The Sensitivity of the Reaction of Dentin to Various Disturbances in Calcium Metabolism: The Calciotraumatic Ring

The dentin that was formed and calcified during and immediately after the operation shows in longitudinal sections a fine sharp line or sometimes a double line which stands out by its deeper and more distinct staining with hematoxylin (Figs. 1, 2). In transverse sections this line takes the appearance of a ring. This ring is not seen in animals of long survival because the dentin formed at the time of the operation has been abraded. This ring is not characteristic of parathyroidectomy alone. It has also been found subsequent to adrenalectomy [14] and hypophysectomy (unpublished data) in the dentin forming and calcifying at the time of the operation.

A similar acute response is also found to be associated with the effects of injections of parathyroid extract [9] and sodium fluoride.[25] The immediate primary hypocalcified zone following the injection of sodium fluoride is often readily demarcated from the preexperimental dentin by a fine narrow hematoxylin staining line which facilitates the recognition of the starting point for measuring the width of the postoperative dentin.

This acute dentin response offers an interesting problem for analysis. It seems to be an expression of a shock to calcium metabolism. This is perhaps induced by the trauma incident to surgery, ether anesthesia, or acute endocrine disturbances. It may be an overcalcification effect associated with a very brief period of arrested growth (Harris [26]). Regardless of the particular etiology of this reaction, we may consider the latter as an experimentally or otherwise induced hypercalcification effect in the dentin. The ring itself might be referred to as the calciotraumatic dentin ring. It illustrates in an accentuated manner the normal dual character of the calcification process of dentin which consists of a rhythmic alternation of dense and less dense mineralization.

An analogous macroscopic condition has been observed in the

roentgenograms of long bones which register transverse lines of arrest [26] or scars [27] in response to various acute disturbances.

Variations in Calcification Disturbances Within the Same Incisor

Erdheim's calcioprotective law emphasizes the fact that disturbances in calcium metabolism do not affect different tissues in the same manner or to the same degree. Our findings indicate that this generalization is also applicable to the different teeth of the same animal and to different portions of the same tooth. The experimental disturbances in the dentin calcification were more severe in the upper than in the lower incisors and were more evident in the lingual than in the labial portions.

It is interesting that even in the most severe alteration in the calcification of the dentin, the portion immediately adjacent to the enamel or cementum (mantle dentin) is not affected. There appear to be localized factors that modify to a limited extent the effect of systemic conditions.

The Validity of Hematoxylin as an Indicator of Calcification

The question of the validity of the hematoxylin-eosin stain used in decalcified sections as an indicator of calcification was discussed in detail in a previous report (Schour and Ham [28]). In this study confirmation of our interpretations on the basis of the staining reaction was obtained by the roentgenograms. Incremental eosin staining zones (Plate 144, Fig. 2) were found to be radiolucent in the roentgenograms (Figs. 1, 4, 7, 8). We have obtained additional confirmatory evidence by the use of Grenz X-rays of ground sections that were kindly taken for us by Dr. E. Applebaum. Further and more conclusive evidence awaits the application of microhardness tests, microchemical analysis and possibly X-ray diffraction studies.

It is hoped that in the near future improved methods and facilities for analysis will clarify further the important problem of interpreting different quantitative and qualitative degrees of calcification in the hard dental tissues.

Summary and Conclusions

The effect of parathyroidectomy on the incisor of the albino rat was studied in 100 rats (3 to 360 days after operation) and 27 controls, in respect to gross changes (including roentgenograms

and eruption rates) and microscopic alterations. Eighteen rats were allowed to survive only 3–20 days (Group I). Fifty-seven rats survived a period of 39 to 159 days (Group II). Fourteen rats survived 105 days and were subjected to fasting every 7th day (Group III). Eleven rats survived approximately 4 months to 1 year, and were subjected to repeated pregnancies and lactations (Group IV).

The incisor of the parathyroidectomized rat shows disturbances in calcification and in development. The essential character of the reaction varies with the length of the survival period.

Group I. In short survivals up to 20 days the postoperative dentin indicates denser calcification than normal and is demarcated from the preoperative dentin by a distinct ring, which stains deeply with hematoxylin. This ring, which is formed at the time of the operation and which is also found in other related experimental conditions, appears to be an acute response to a shock to calcium metabolism and may be referred to as the calciotraumatic ring. The denser calcification of the postoperative dentin in this group may be a result of the calcium retention that is reported to persist during the early survival period.

Group II. In longer survivals the changes consist of defective calcification and defective formation of enamel and dentin. The severity of the changes increases with the increase in time of survival. The most characteristic change consists of an aberration in dentin calcification. The dentin shows an irregular and accentuated alternation of zones of different densities in calcification. This severe fluctuation, rather than lack of calcification, is responsible for the readiness of the parathyroprivic dentin to fracture.

Group III. The histological changes are aggravated by fasting on every 7th day.

Group IV. The most severe alterations were found in animals that were subjected to repeated pregnancies and lactations. Histological examination shows no evidence of calcium withdrawal from the calcified tissues of the teeth.

Our findings differ from those reported in the literature in the following respects: dense calcification of dentin during the early survival period (Group I); less severe changes for same period of survival (Group II); and a less prominent rachitic-like width of predentin. These differences may be ascribed chiefly to a difference in diet.

REFERENCES

1. Erdheim, J. Tetania parathyreopriva. *Mitt. a. d. Grenzgeb. d. Med. u. Chir.*, 1906, **16**, 632–744.
2. Toyofuku, T. Ueber die parathyreoprive Veränderung des Rattenzahnes. *Frankfurt. Ztschr. f. Path.*, 1911, **7**, 249–294.
3. Preiswerk-Maggi, P. Ueber den Einfluss der Parathyroidektomie auf die Nagezähne der Ratten. *Deutsche Monatschr. f. Zahnh.*, 1911, **29**, 641–680.
4. Von Spreter, T. Experimentelle Untersuchungen über die Wirkung des bestrahlten Ergosterins auf die parathyreoprive Ratte. *Ztschr. f. d. ges. exper. Med.*, 1932, **85**, 19–45.
5. Erdheim, J. Über die Dentinverkalkung im Nagezahn bei der Epithelkörperchentransplantation. *Frankfurt. Ztschr. f. Path.*, 1911, **7**, 295–342.
6. Erdheim, J. Rachitis und Epithelkörperchen. *Denkschr. d. math. naturw. Klasse d. Kaiserl. Akad. d. Wissensch.*, Wien, 1914, **90**, 363–683.
7. Schour, I., Tweedy, W. R., Chandler, S. B., and Engel, M. B. Changes in teeth following parathyroidectomy. II. The effect of parathyroid extract and calciferol on the incisor of the rat. *Am. J. Path.*, 1937, **13**, 971–984.
8. Chandler, Simon. The relation of parathyroidectomy to estrus, pregnancy, and lactation in the albino rat. *Anat. Record*, 1932, **53**, 105–120.
9. Schour, I., Tweedy, W. R., and McJunkin, F. A. The effect of single and multiple doses of the parathyroid hormone on the calcification of the dentin of the rat incisor. *Am. J. Path.*, 1934, **10**, 321–342.
10. Schour, I., and van Dyke, H. B. Changes in the teeth following hypophysectomy. I. Changes in the incisor of the white rat. *Am. J. Anat.*, 1932, **50**, 397–433.
11. Schour, Isaac, and Steadman, Sherwood R. The growth pattern and daily rhythm of the incisor of the rat. *Anat. Record*, 1935, **63**, 325–333.
12. Schour, Isaac, and Hoffman, M. M. Demonstration of 16 micra rhythm in the normal stratification of enamel and dentin in man and other mammals. *J. Dental Research*, 1935, **15**, 161.
13. Schour, Isaac, and Hoffman, M. M. Experimental demonstration of daily apposition of 16 micra of enamel and dentin in growing mammalian teeth. *J. Dental Research*, 1935, **15**, 161–162.
14. Schour, I., and Rogoff, J. M. Changes in rat incisor following bilateral adrenalectomy. *Am. J. Physiol.*, 1936, **115**, 334–344.
15. Gottlieb, B. Schmelzhypoplasie und Rachitis. *Wien. Vrtljschr. f. Zahnh.*, 1920, **36**, 76–96.
16. Von Spreter, T. Experimentelle Untersuchungen uber den Rattennagezahn als Testobject für die Wirkung des Epithelkörperchenhormons. *Deutsche Zahn-, Mund- und Kiefer-Zahnh.*, 1936, **3**, 318–331.
17. Hoskins, Margaret M., and Chandler, Simon B. Accessory parathyroids in the rat. *Anat. Record*, 1925, **30**, 95–98.

18. Hoskins, Margaret M. Variations in the susceptibility of the albino rat to tetania parathyropriva. *Endocrinology*, 1931, **15**, 324–334.

19. Shelling, David H. The Parathyroids in Health and in Disease. C. V. Mosby Company, St. Louis, 1935.

20. Shelling, David H. Effect of diet and viosterol on calcium deposition in the callus of parathyroidectomized rats. *Proc. Soc. Exper. Biol. & Med.*, 1930, **28**, 306–307.

21. Von Korenchevsky. The influence of parathyroidectomy on the skeleton of animals normally nourished and on rickets and osteomalacia produced by deficient diet. *J. Path. & Bact.*, 1922, **25**, 366–392.

22. Von Spreter, T. Weitere experimentelle Untersuchungen über die Auswirkung der Exstirpation der Epithelkörperchen und des Thymus bei Ratten auf die Entwicklung der Nagezähne und ihre Beeinflussung durch Vitamin D. *Ztschr. f. d. des. exper. Med.*, 1935, **96**, 95–115.

23. Bülbring, E. Über die Beziehungen zwischen Epithelkörperchen, Calciumstoffwechsel und Knochenwachstum. *Arch. f. exper. Path. u. Pharmakol.*, 1931, **162**, 209–248.

24. Albright, Fuller, Aub, Joseph C., and Bauer, Walter. Hyperparathyroidism: a common and polymorphic condition as illustrated by seventeen proved cases from one clinic. *J. A. M. A.*, 1934, **102**, 1276.

25. Schour, I., and Smith, M. C. The histologic changes in enamel and dentin of the rat incisors in acute and chronic experimental fluorosis. *Univ. Arizona Agric. Exper. Stat., Tech. Bull.*, 1934, No. 52, 69–91.

26. Harris, H. A. Bone Growth in Health and Disease. Oxford University Press, London, 1933.

27. Todd, T. W. Mutilated growth. *J. Am. Dent. A.*, 1934, **21**, 479–488.

28. Schour, I., and Ham, A. W. The action of vitamin D and of the parathyroid hormone on the calcium metabolism as interpreted by studying the effect of single doses on the calcification of dentin. *Arch. Path.*, 1934, **17**, 22–39.

DESCRIPTION OF PLATES

PLATE 142

Fig. 1. Microphotograph of a longitudinal section from the midregion of the upper right incisor of Rat 55 which was killed 8 days after parathyroidectomy. Note the deep hematoxylin staining line (l) at the level of the dentin which was calcifying at the time of operation and which separates the preoperative dentin (D. pr.) from the postoperative dentin (D. po.). The latter takes a deeper stain with hematoxylin. Measurements of the distance of the line l from the predentin show that the position of the line corresponds closely with the position of the dentin that was being calcified about the time of the operation. En. m. = enamel matrix; L. a. p. = labial alveolar periosteum; Od. = odontoblasts. × 84.

Fig 2. Microphotograph of a longitudinal section from the midregion of the lingual half of the upper right incisor of the same animal as in Figure 1. The deeper hematoxylin staining line (l) is followed pulpally by a

lighter staining zone (z) and separates the preoperative (D. pr.) from the postoperative dentin (D. po.). The calcification is modified after the operation, as is evidenced by the deeper staining reaction. Cem. = cementum; Od. = odontoblasts; P. d. m. = periodontal membrane. × 84.

Fig. 3. Roentgenogram of right half of the head of an adult control rat. Note the smooth curvature of the tooth surfaces. The distal third or half of the tooth is radiopaque while the radiolucent pulp can be seen more posteriorly. Figures 3–10 are natural size.

Fig. 4. Roentgenogram of the upper and lower incisors of parathyroidectomized Rat 120 of Group II. Note the irregular contour of the labial outline of the upper incisor which is found in about 30 per cent of the animals in this group. The radiolucent appearance of the pulp persists as far as the alveolar crest. In the lingual dentin of the lower incisor, near the alveolar crest, there is an incremental radiolucent zone between two radiopaque borders. This zone corresponds to the eosin staining areas seen in the microscopic sections. The lower incisor is elongated because its antagonist had been fractured.

Fig. 5. Roentgenogram of Rat 10A of Group II. The upper incisor shows an intra-alveolar fracture and a wide pulp. In the lower incisor the radiolucent appearance of the pulp extends to the incisal edge.

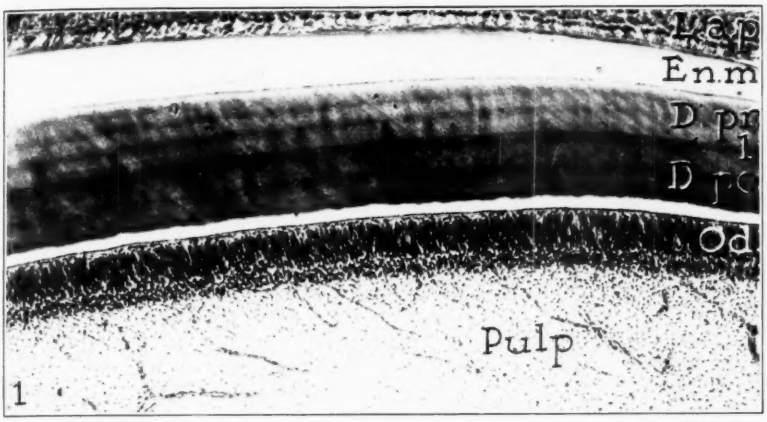
Fig. 6. Roentgenogram of the upper right incisor of Rat 119 of Group II. Note the irregular contour of the enamel surface and the extent of the pulp to the incisal edge. The notch in the enamel near its distal end represents the marking filed into the tooth for the purpose of measuring the rate of eruption.

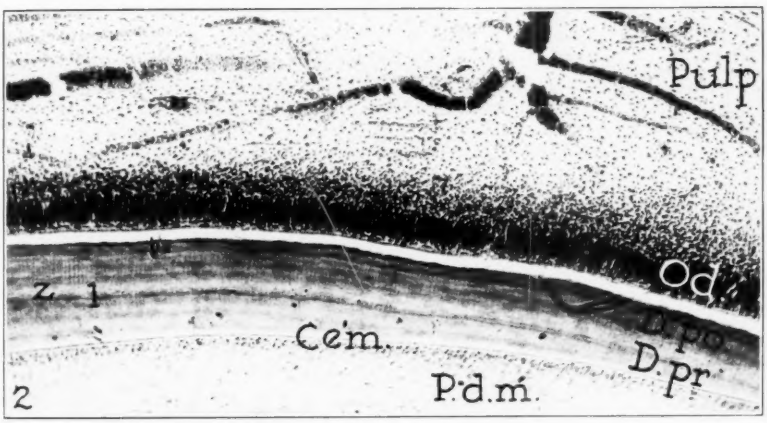
Fig. 7. Roentgenogram of the left half of the head of Rat 709 of Group IV. Note in the upper incisor the elongation; the irregular contour of the enamel surface; the irregular width of the labial alveolar periosteum; and the abnormal width of the pulp and the persistence of its radiolucency beyond the alveolar crest. The lower incisor shows an incremental radiolucent zone in the distal half of its lingual portion and an intra-alveolar fracture.

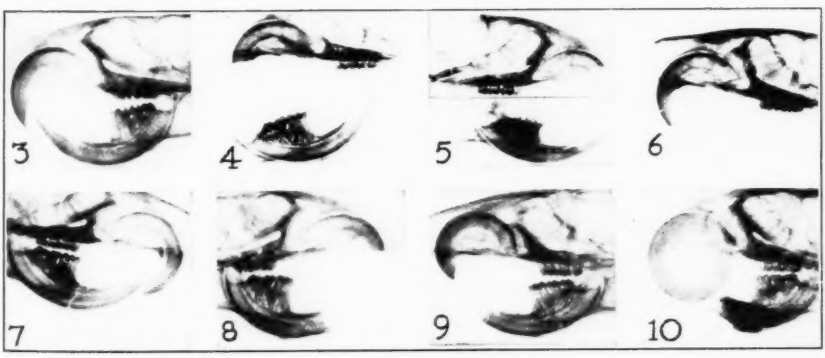
Fig. 8. Roentgenogram of the right half of the head of Rat 704 of Group IV. Note in the upper incisor the irregular enamel surface, the irregular width of the labial alveolar periosteum and the fracture. Note in the lower incisor the intra-alveolar fractures and the lingual incremental radiolucent zone in the fractured segment.

Fig. 9. Roentgenogram of the left half of the head of the same rat as in Figure 8. Note in the upper incisor the distortion of the curvature and the severe enamel hypoplasia in the basal area. The pulp is radiolucent only in the proximal half. The lower incisor is thickened in the proximal end of the labial surface and shows a fracture.

Fig. 10. Roentgenogram of the right half of the head of Rat 712 of Group IV. The changes are characteristic of Group IV. Note the elongation of the upper incisor through the lack of function subsequent to the fracture of the lower tooth. Note the perforation of the palate at the distal end of the upper incisor which almost meets its proximal beginning. The dentin shows radiolucent zones. The lower incisor shows a thickening of its proximal labial end.







Schour, Chandler and Tweedy Changes in Teeth Following Parathyroidectomy. I.

PLATE 143

Fig. 11. Microphotograph of a transverse ground cross section of the lower left incisor of Rat 64 which survived 157 days after parathyroidectomy. It received 1 injection of parathyroid extract 3 days before death. Note the two prominent concentric rings of interglobular dentin (Int.). The inner ring is absent where the dentin (D. po.) is covered by the enamel. The missing dentin was fractured in grinding along the poorly calcified incremental zone (Y.). The enamel (En.) shows a lack of homogeneous calcification. The labial alveolar bone (Al. b.) at the left shows interesting rhythmic calcification zones. L. a. p. = labial alveolar periosteum; P. d. m. = periodontal membrane; Cem. = cementum. Pulp torn out in grinding. × 23.

Fig. 12. Microphotograph of a longitudinal ground section from the midregion of upper incisor of Rat 709 which was parathyroidectomized and subjected to repeated pregnancies and lactations. Note the wavy surface and prominent incremental stratification in the enamel (En.) and the zone of interglobular dentin (Int.) in the postoperative dentin (D. po.). × 60.

Fig. 13. Microphotograph of a longitudinal section from the distal portion of the labial dentin of the upper left incisor of Rat H50 which lived 105 days after parathyroidectomy and was subjected to fasting every 7th day. Note the wave-like disturbance of the dentino-enamel junction and the enamel surface. The parathyroprivic dentin (D. po.) shows the typical irregular calcification zoning. The enamel epithelium is severely atrophied. L. a. p. = labial alveolar periosteum; En. sp. = enamel space. Od. = odontobalsts. × 34.

Fig. 14. Microphotograph of a longitudinal section from the midregion of the labial dentin of the upper right incisor of Rat 116 which lived 120 days after parathyroidectomy and was given 1 administration of calciferol 24 hours before death. Note the downgrowth of the labial alveolar periosteum (L. a. p.) and the hemorrhage in the pulp (h) at the fracture (Fr.) showing that the latter is not an artefact; the alternate zones of varying degrees of disturbances in calcification; and the bending of the zones at the sides of the vascular inclusion (V. i.). En. sp. = enamel space; D. m. = mantle dentin; D. po. = postoperative dentin; X = cyst in odontoblastic layer. × 62.5.

Fig. 15. Microphotograph of a longitudinal section from the labial dentin of the upper incisor of a control rat. Note the uniform density of calcification and the normal calcification rhythm. Compare with the parathyroprivic dentin in Figures 13 or 14 of this plate. En. sp. = enamel space; D = dentin; Pre = predentin; Od. = odontoblasts. × 100.

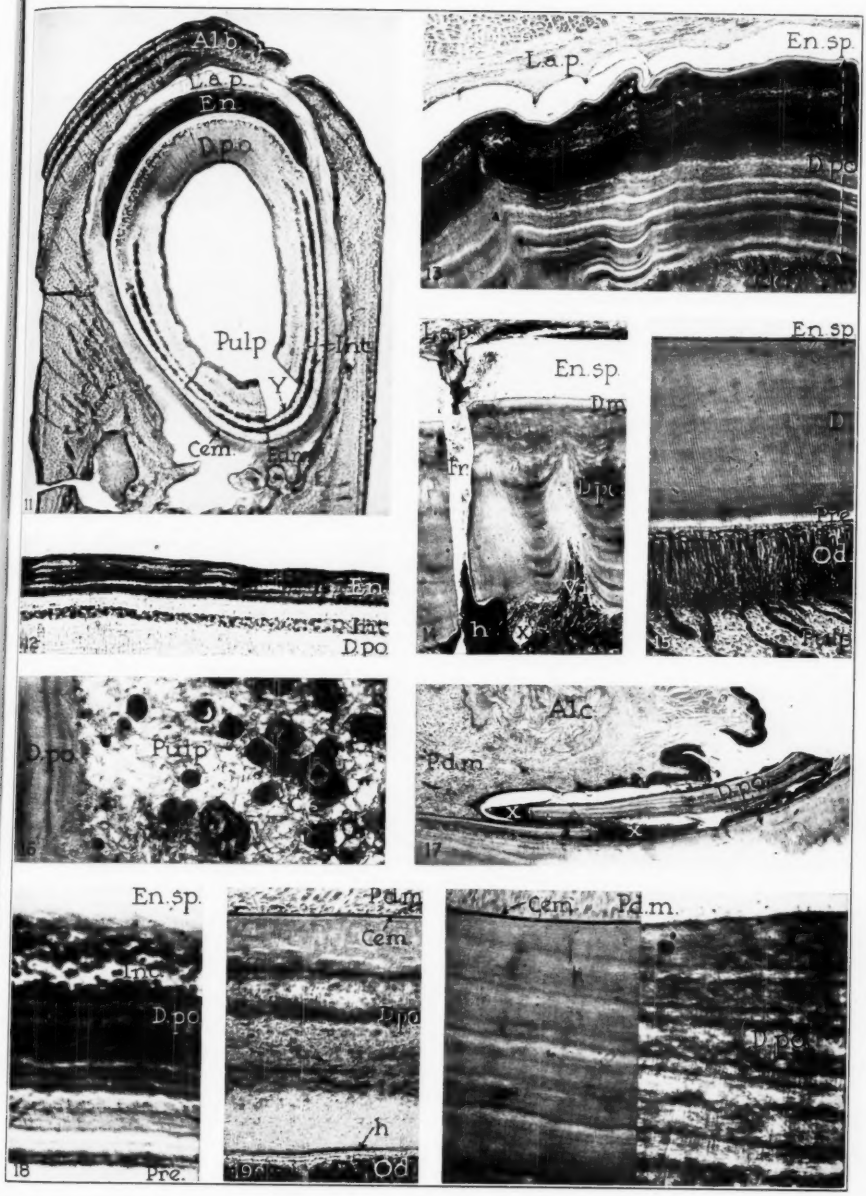
Fig. 16. Microphotograph of a longitudinal section from the incisal portion of the pulp of the upper incisor of Rat 119 of Group II. Note the complete atrophy of the pulp and the numerous calcospherites. D. po. = postoperative dentin; C. s. = calcospherites. × 115.

Fig. 17. Microphotograph of a longitudinal section from the incisal portion of the lingual dentin of the upper right incisor of Rat 126 which lived 133 days after parathyroidectomy and was given 1 injection of parathyroid extract 240 hours before death. The dentin fractured along an incremental line of disturbed calcification. Contrast with the type of fracture seen in Figure 14. Note the proliferation of the alveolar bone at the crest (Al. c.); the downgrowth of epithelium along the fractured surface which faces the periodontal membrane and the cellular débris collected at X. D. po. = postoperative dentin; P. d. m. = periodontal membrane. × 22.5.

Fig. 18. Microphotograph of portion of a longitudinal section from the midlabial dentin of Rat H53 of Group III. Note the interglobular dentin (Int.) and the calcification zones of different widths and densities in the parathyroprivic dentin (D. po.). En. sp. = enamel space; Pre. = predentin. × 90.

Fig. 19. Microphotograph of a longitudinal section from the midregion of the lingual dentin of the lower right incisor of Rat 113, which lived 120 days after parathyroidectomy and was given 1 administration of calciferol 24 hours before death. D. po. = dentin laid down previous to the administration. Note the alternate zones of varying degrees of calcification disturbances, particularly the uncalcified zone formed preceding the time of administration. Note the very narrow hematoxylin staining zone (h) immediately next to the narrow predentin. This zone was produced by the administration of calciferol. Cem. = cementum; Od. = odontoblasts; P. d. m. = periodontal membrane. × 100.

Fig. 20. Microphotograph of a longitudinal section from the lingual dentin of Rat H40 of Group III. Note the prominent zoning of eosin or hematoxylin staining dentin and the irregularity in width and staining reaction of these zones. Fasting on every 7th day tended to accentuate the zoning effect. Cem. = cementum; P. d. m. = periodontal membrane; D. po. = postoperative dentin. The right and left portion of the figure show one field but the photographs were taken with different filter and focus to show interglobular texture and irregular stratification pattern. × 180.



Schour, Chandler and Tweedy

Changes in Teeth Following Parathyroidectomy. I.

CHANGES IN THE TEETH FOLLOWING PARATHYROIDECTOMY *

II. The Effect of Parathyroid Extract and Calciferol on the Incisor of the Rat

I. Schour, D.D.S., Ph.D., W. R. Tweedy, Ph.D., S. B. Chandler, M.D., and M. B. Engel

(From the Department of Histology, University of Illinois College of Dentistry, and the Departments of Biochemistry and Anatomy, Loyola University School of Medicine, Chicago, Ill.)

This study is concerned with the evaluation and comparison of the effects of parathyroid hormone and of calciferol (vitamin D) on the incisors of parathyroidectomized rats.†

Review of Literature

Von Spreter [1] administered variable doses of parathyroid hormone to 20 rats within 12 days, or less, after parathyroidectomy. He found that a minimum of 20 to 24 (Collip) units of parathyroid extract (Lilly) was necessary to produce complete calcification of the "dentinoid" tissue and that the effect of a single injection persisted for 12 to 24 hours. Schour and Ham,[2] and Schour, Tweedy, and McJunkin [3] reported the effects of variable, single or multiple, doses of vitamin D, and of parathyroid hormone on the serum calcium and incisors of normal rats. They found that a single effective dose of either vitamin D or parathyroid hormone produced in the dentin a primary hypocalcified stripe which was followed by a hypercalcified stripe.

Von Spreter [4] treated 3 parathyroidectomized rats with daily doses of 150 rat units of irradiated ergosterol, and 3 parathyroidectomized rats with daily doses of 5000 rat units of irradiated ergosterol. In each case the administration was begun after the appearance of tetany. The doses of 150 rat units of irradiated ergosterol had no effect. The doses of 5000 rat units prevented and healed the parathyroprivic defects in the teeth, without metastatic calcification or other symptoms of overdose.

* This investigation was aided by a grant to one of us (I. S.) from the Committee on Scientific Research of the American Medical Association and from the Graduate School Research Board of the University of Illinois.

† A portion of the parathyroid extract was supplied through the courtesy of Eli Lilly and Company. We are indebted to Mead Johnson and Company for the calciferol used in these experiments.

Received for publication July 15, 1937.

Methods and Material

This report is based on observations of 59 parathyroidectomized albino rats. Twenty-nine of these animals received from 1 to 4 injections of either Lilly's parathyroid extract or of the extract prepared by one of us (W. R. T.). The total dosage ranged from 10 to 274 (Collip) units (Table I). Single doses of 46,000 to 644,000 international units of calciferol were administered orally to 30 animals (Table II). The calciferol was in solution in Mazola

Table I

Data on 29 Parathyroidectomized Rats Treated with Parathyroid Extract, Arranged According to Survival Period

Rat No.	Post-operative survival to injection time	Weight at injection	Collip units	Number of injections	Interval between first injection and death	Calculated survival
	days	*gm.*	*per 100 gm.*		*days*	*days*
2	66	..	50	1	3	2.6
7	66	158	50	1	3	2.6
3	66	247	50	1	3	2.8
1	66	224	50	1	3	2.9
37	20	145	10	1	3	2.9
38	20	147	10	1	3	3.0
64	157	215	25	1	3	3.0
32	98	290	25	1	4	3.7
66	157	163	50	1	4	3.8
96	157	274	25	4 [1]	4	3.9
120	125	220	25	4 [1]	4	3.9
48	47	155	25	1	4	4.0
29	98	257	25	1	4	4.1
904*	83	167	10	1	4	3.7
907*	83		10	1	4	3.7
903*	83	to	10	1	4	3.8
905*	83		10	1	4	4.2
901*	83	298	10	1	4	4.3
906*	83		10	1	4	4.4
119	125	241	25	4 [1]	5	5.0
100	140	190	50	1	8	8.2
101	140	240	50	1	8	8.4
30	98	298	25	1	9	8.8
67	39	156	50	1	9	8.9
112	135	228	25	2 [2]	11	10.8
108	135	215	25	2 [2]	11	11.3
111	135	188	25	2 [2]	11	11.6
68	39	173	50	1	17	17.1
47	47	160	100	1	17	17.3

* History of animals marked 900 or above was available for entire group only.
[1] Interval between injections, 1 day.
[2] Interval between injections, 3 days.

TABLE II

Effect of Calciferol on the Serum Calcium of Parathyroidectomized Rats, Arranged According to Survival Period After Administration

Rat No.	Post-operative survival to first administration	Weight at administration	Total dose in I.U. ($\times 10^3$)	Post-administration survival	Serum calcium (just before sacrifice)	
	days	*gm.*		*hrs.*	*mg./100 cc.*	
*118	120	176	368	24	12.21	Pooled sample
113	120	233	552	24	12.21	
116	120	267	644	24	12.21	
1A	58	148	184	48	13.60	Pooled sample
3A	58	152	276	48	13.60	
5A	57	190	378	48	13.60	
91	159	207	460	72	16.04	Pooled sample
92	159	217	460	72	16.04	
93	159	186	368	72	16.04	
13A	56	159	276	96	14.18	Pooled sample
14A	56	182	368	96	14.18	
17A	55	124	184	96	14.18	
25A	42	175	368	120	13.18	Pooled sample
*26A	42	170	276	120	13.18	
27A	42	124	184	120	13.18	
4A	57	207	460	144	11.63	Pooled sample
6A	56	217	460	144	11.63	
10A	56	186	368	144	11.63	
125	133	168	368	168	9.23	Individual sample
*124	133	142	184	216	7.79	
126	133	165	276	240	8.81	
Administration of Small Doses of Calciferol to Parathyroidectomized Rats						
*1B	49	150	46	66	10.46	
†3B	49	141	46	66		
*4B	49	220	115	66		
5B	49	249	138	66	13.37	
6B	48	220	115	66	11.34	
†7B	48	282	161	66	11.44	
*8B	48	175	92	66		
†9B	48	182	92	66	11.19	Pooled sample
10B	48	170	69	66	11.19	

* These rats were not studied histologically.

† In the cases of animals 3B, 7B, and 9B, the serum calcium 17 hours after injection was 8.62, 9.60 and 9.77 mg. per cent respectively.

oil. The untreated parathyroidectomized rats included in the preceding report were used as controls. Therapy was instituted at 20 to 159 days following the operation and the animals were sacrificed 3 to 17 days after the administration of calciferol or the last injection of parathyroid hormone. The histological technique was the same as that used in the preceding report.[5]

Chemical Findings

Serum calcium values were obtained immediately after sacrifice of each parathyroidectomized animal to which calciferol was administered (Table II). There appears to be a correlation between the serum calcium values, the duration of the postadministration

Table III

Effect of Calciferol on the Serum Calcium of Normal Rats

Rat No.	Weight at administration	Total dose in I.U. ($\times 10^3$)	Post-administration survival	Serum calcium (just before sacrifice)	
	gm.		*hrs.*	*mg./100 cc.*	
1	133	184	48	15.23	Pooled sample
3	173	368	48	15.23	
5	139	184	48	15.23	
2	156	276	48	15.35	Pooled sample
4	213	460	48	15.35	

period and the dosage. The serum calcium tends to rise during the first 3 days following the injection and then begins to decline thereafter. The level and rapidity of the rise appears to increase with the dosage. In the series of 9 parathyroidectomized rats that were given one-fourth the dosage of calciferol and in which serum calcium levels were obtained 17 hours and 66 hours after the administration, the elevation of serum calcium was not so pronounced. Table III also indicates the serum calcium values obtained in a series of 5 normal rats that were given calciferol and were allowed to live 48 hours. The calcium level is higher than in the corresponding group of parathyroidectomized rats that were given approximately the same dosage per 100 gm. of body weight and killed at the same time interval.

Histological Findings

Effect of a Single Injection of Parathyroid Extract

The dosage of parathyroid hormone administered in single injections varied from 10 to 100 (Collip) units per 100 gm. of body weight. The postinjection survivals ranged from 3 to 17 days.

Hormone therapy results in an improvement in the calcification of the incisor. The somewhat wider predentin of the parathyroprivic animals is no longer present. The injection also has a characteristic effect on the dentin (Figs. 1, 2 and 4). The dentin which is forming and calcifying at the time is sometimes sharply demarcated against the preinjection dentin by a fine distinct hematoxylin staining line. The primary effect is a hypocalcified eosin stripe. The secondary effect is a hematoxylin staining zone which extends to the intermediate dentin border. This hematoxylin assumes a characteristic blue color, the intensity of which varies in different animals (Figs. 3, 4 and 6). The total effect also varies in different areas of the incisor. Thus, in the proximal portion, the eosin stripe is wider and the hematoxylin area is narrower. Nevertheless, the primary and secondary effects attain a constant width in the middle third in both the labial and lingual portions of the dentin.

Rats 37 and 38 both received 14.5 units on the 20th day after operation and were killed 3 days after administration of the hormone. Micrometer measurements indicate typical injection zones of 2.9 and 3 days respectively. The primary hypocalcified response however is very weak.

When higher doses of hormone are administered a more prominent reaction is often induced. This is indicated in Rat 29, which received 64.2 units 98 days after operation. The effect was most pronounced on the lingual portion of the upper incisor where a very heavy secondary hematoxylin stripe was noted (Figs. 1, 2 and 4). This zone is 66 μ wide, which indicates 4.1 days of formation. The postinjection survival was 4 days.

Our experiment includes a series of 6 animals surviving the injection for periods longer than 4 days (Table I). In this group the injection effect persists either for the entire survival periods (Fig. 3), or for shorter periods, so that the dentin returns gradually to the condition of disturbed calcification preceding therapy.

In such instances there is a sharp indication of the cessation of the therapeutic effect. In Rat 68, which had a survival period of 17 days, the typical primary and secondary response affected a zone of dentin formed during the first few days, but the dentin formed subsequently showed what appeared to be normal calcification for the remainder of the survival period.

Effect of Multiple Injections of Parathyroid Hormone

Six parathyroidectomized animals were given multiple injections of parathyroid extract, the total dosage ranging from 94 to 274 (Collip) units (Table I). The effect produced was usually the same as that resulting from a single injection, *i.e.* an eosin stripe which constituted the primary response to the first injection, followed by a hematoxylin zone corresponding to the secondary response of the first injection, and the combined effect of the remaining injections.

In Rats 111 and 108 the last injection was administered 7 days before death. The effect is noted to persist until death (Fig. 6). However, in Rat 112, which also belongs to the same group and received a similar dosage, there is a return to the disturbed parathyroprivic condition before death. The fact that the dentin was more severely disturbed following parathyroidectomy in Rat 112 than in the other 2 animals may account for this observation.

Table I shows the close agreement between the calculated survival (last column) and the actual survival after the first injection (column before the last). The calculated survival was obtained by measuring in microns the total width of the primary eosin effect and the secondary hematoxylin effect and dividing the amount by 16. Sixteen microns represents the average daily growth of dentin.

In 16 animals of the group treated with parathyroid hormone a definite cytological change was observed in the active ganoblasts which were situated at the level of the middle third of the enamel matrix. At their distal portion adjacent to the enamel matrix, clear globular inclusions were noted (Fig. 5). These were not seen in normal animals treated with parathyroid extract or in animals that were only parathyroidectomized.

Neither an abnormal number of osteoclasts nor osteitis fibrosa was detected in the parathyroprivic rats that were treated with parathyroid extract in the amounts indicated (Table I).

Replacement Therapy with Effect of Single Administrations of Calciferol (Vitamin D)

In this series 30 animals were given oral administrations of calciferol in doses ranging from 46,000 to 644,000 international units. The animals were treated at 42 to 159 days following parathyroidectomy (Table II). The postadministration period was from 1 to 10 days. The dentin reaction is similar to that obtained when parathyroid extract is administered (Figs. 8 and 9). Also, in 13 animals of this group the same type of cytological reaction in the ganoblasts as is described above was observed.

In the group that received smaller doses of calciferol (Table II) it was difficult to detect a response in the dentin and no inclusions in the ganoblasts were noted.

Rat 126 received 276,000 international units and was permitted to live 10 days following administration. Histological examination shows that the secondary hypercalcification effect on the dentin persisted for 10 days. Similar persistence of effect was noted in Rat 125 which survived 7 days.

The most delicate reaction was observed in Rats 113 and 116. These animals received respectively 552,000 and 644,000 units of calciferol. They were both killed 1 day after treatment. Careful histological examination showed a narrow hematoxylin staining line next to the pulp with an intervening narrow predentin border (Fig. 7). The position of the experimental hematoxylin border indicates that it was produced at about the time of the treatment. The inclusions in the ganoblasts noted in other animals were also present here.

Discussion

Effect of Replacement Therapy with Parathyroid Extract

The effect of parathyroid extract on parathyroprivic dentin is of the same type as that observed by Schour and Ham,[2] and Schour, Tweedy and McJunkin [3] in normal dentin, namely a short primary hypocalcified reaction and a secondary overcalcified reaction. However, doses (10 Collip units per 100 gm. of weight) which were ineffective in normal animals were sufficient to produce the typical response in the experimental animals and to reduce the predentin width in the parathyroprivic dentin.

The secondary reaction effected an overcalcification or recovery which tended to continue throughout the survival periods that were tested (1–10 days). In some instances of survival of longer than 6 days the deeper staining reaction with hematoxylin receded during the later portion of the survival period and blended into the staining reaction of a normally calcified dentin.

We were able to calculate the time interval that elapsed between the time of the first injection and the death of the animal by measuring the width of the parathyroprivic dentin that was affected by the administration of the parathyroid hormone in μ and dividing this amount by 16 (which represents the number of μ of dentin per day laid down normally in the incisor of the rat). This estimate was found to correspond to the actual time of survival with an average accuracy of one-half of 1 day. The rate of apposition of the experimental dentin is therefore similar to that of normal dentin. These findings are in disagreement with those of Von Spreter,[1] who reports a rate of apposition of 8 to 12 μ per 24 hours and who accepts 10 μ as the normal daily rate of dentin apposition.

The explanation of the primary and secondary reaction might be made tentatively on the basis of the change in concentration of serum calcium. There is the possibility of obtaining a beautiful correlation if the serum calcium and phosphorus can be determined at a number of intervals before death. As in the case of experimental hyperparathyroidism in normal rats,[2, 3] the primary hypocalcified dentin zone is perhaps associated with a period of calcium mobilization which results in an increased calcium level in the blood. The secondary hypercalcification effect is perhaps associated with the lowering of the serum calcium.

Some of the histological data do not support this possible correlation between the histological picture and the chemical reaction. The extent of the hypocalcified stripe usually indicates a shorter chronological duration than the period of the rise in calcium. The width of the primary reaction possibly corresponds more nearly to the period concerned with the initial disturbances incident to the time of active mobilization. It is also possible that the extent of the primary reaction is reduced by the process of secondary calcification that may be especially effective during the period of the decline of serum calcium.

The present as well as previous studies indicate clearly that the

dentin reaction differs from that of bone. Von Spreter [1] was unable to demonstrate osteitis fibrosa and abnormal osteoclastic activity in parathyroprivic animals. Our findings confirm this observation. The absence of the established histological symptoms of osteitis fibrosa in experimental hyperparathyroidism may be attributable to the fact that the dosage of parathyroid extract was insufficient both to compensate for the parathyroprivic conditions and to produce symptoms of overdosage.

Effect of Calciferol on the Parathyroprivic Dentin: A Comparison with the Effects of Parathyroid Extract

In our study no differentiation between therapy with calciferol and parathyroid extract was noted; the same primary and secondary reaction in the dentin occurred in the animals injected with parathyroid extract as in the animals treated with calciferol. Table II indicates a correlation between our histological findings following the administration of calciferol and the serum calcium values. The latter appear to be similar although slightly lower than those found in hypervitaminosis D in normal rats.[2]

The portion of the postinjection dentin which shows the primary hypocalcified reaction is as a rule narrower than the portion showing the secondary hematoxylin staining reaction. This is perhaps associated with the fact that the rise of the serum calcium extends over a shorter period of time than its subsequent decline to the original level. The width of the dentin showing the primary reaction is less than the amount of dentin that formed during the duration of the serum calcium rise. Here, as in the case of the injections of parathyroid extract, it is possible that the original width of the dentin involved in the primary response was greater but became partly obliterated by a process of secondary calcification that may have occurred during the duration of the secondary reaction.

The cytological inclusions in the enamel-forming cells suggest an increased sensitivity of these cells to parathyroid extract and calciferol.

The mechanism of action of vitamin D has as yet not been determined. Taylor, Weld, Branion and Kay [6] postulate an action through stimulation of the parathyroids. They report negative results with concentrated vitamin D in attempts to alleviate para-

thyropriva tetany. Success of other workers is attributed to the presence of accessory parathyroid rests which can only be removed by a free dissection of the neck. Jones [7] was unable to raise the serum calcium level in parathyroidectomized dogs with daily doses of 20 cc. of cod liver oil.

Von Spreter [4] does not regard irradiated ergosterol as a substitute for parathyroid extract and suggests the possibility that the irradiated ergosterol exerts its effect through the accessory parathyroids or other organs sensitive to vitamin D, or through compensating endocrine glands.

Hess, Weinstock and Rivkin [8] succeeded in raising the serum calcium of parathyroidectomized animals to non-tetanic levels by using 100 times the therapeutic dose of irradiated ergosterol or more. Shelling,[9] using very large doses of viosterol, was able to raise the calcium level in parathyroidectomized rats from tetanic to hypercalcemic levels, even on diets containing no calcium or no calcium and phosphorus. It has been suggested that the action of vitamin D is to improve calcium absorption. On the basis of Shelling's findings with diets containing no calcium one would be led to conclude that the mobilization of calcium from the bone by vitamin D given in massive doses occurs independently of its therapeutic effect in increasing absorption. At this juncture, it would be well to indicate that much of the confusion about vitamin D action is caused by the failure of investigators to distinguish between the action of therapeutic and massive doses.

Recently, one of us and coworkers [10] have shown that injection of massive doses of calciferol were ineffective in increasing the serum calcium of the nephrectomized rat, which had previously been thyroparathyroidectomized, but were effective in increasing the serum calcium after nephrectomy alone.

We interpret our results to indicate that with the doses of calciferol employed in this study the dentin response in the parathyroidectomized animal is the same as that obtained with parathyroid extract. This finding concurs with that of those who believe massive doses of vitamin D do not necessitate the presence of the parathyroid in an otherwise intact animal in order to exert their effects.

Duration of the Effect of Single Doses of Parathyroid Extract or Calciferol

Our findings of prolonged improved effects on the dentin calcification for as long as 10 to 17 days following a single administration suggest the advisability of an experimental study on the duration effects of single administrations of therapeutic doses of calciferol. If single therapeutic doses produce prolonged effects for comparable durations, the danger of overdose by the accumulated effect of daily administrations would have to be considered.

Our findings of a 3 to 4 day duration effect of single administration of 14–20 units of parathyroid extract differ from those of von Spreter [1] who reports for this dosage a maximal duration effect of 24 hours.

The Calciotraumatic Ring

The acute reaction that was found to be registered in the calcification of the dentin at the border of the preexperimental and postexperimental dentin in the rats studied in the preceding report [5] was also present in many of the animals in this study (Fig. 7). An examination of the microphotographs published by von Spreter [1] on the effect of injections of parathyroid extract on the parathyroprivic dentin shows the calciotraumatic ring very distinctly. Erdheim's [11] transplantation experiments also show evidences of the calciotraumatic ring.

Summary and Conclusions

This study is based on 59 parathyroidectomized rats that were operated on between the ages of 21 and 93 days. The animals were divided into 2 groups. In Group I, 29 received 1 to 4 injections of parathyroid extract. The total dosage ranged from 10 to 274 (Collip) units. The survival period was from 3 to 17 days. Of Group II, 30 received a single administration of 46,000 to 644,000 international units of calciferol. The survival period was from 1 to 10 days. Serum calcium values were obtained immediately preceding death.

The histological findings in the dentin of the incisors of these animals were similar in Groups I and II and consisted of: (*a*) a primary response characterized by an eosin staining zone; (*b*) a secondary response in the form of a hematoxylin staining zone;

(*c*) in the majority of cases a sharp hematoxylin staining ring at the border of the preexperimental and postexperimental dentin; and (*d*) cytological changes in the active enamel-forming cells in one-half of the animals.

In Group II the serum calcium was found to rise during the first 3 postadministration days and then to decline more slowly to the original level. The level and rapidity of the rise increased with the dosage. The serum calcium values were lower than those found in corresponding conditions in the unoperated animals. The histological effect of single doses lasted as long as 10 days in animals treated with parathyroid extract and calciferol. In 2 rats treated with parathyroid extract the effect lasted 17 days.

Our findings confirm previous data that massive doses of calciferol do not necessitate the presence of the parathyroids in order to exert their effects.

REFERENCES

1. Von Spreter, T. Experimentelle Untersuchungen über den Rattennagezahn als Testobject für die Wirkung des Epithelkörperchenhormons. *Deutsche Zahn-, Mund-, und Kiefer-Zahnh.*, 1936, **3**, 318–331.
2. Schour, Isaac, and Ham, Arthur W. The action of vitamin D and of the parathyroid hormone on the calcium metabolism as interpreted by studying the effect of single doses on the calcification of dentin. *Arch. Path.*, 1934, **17**, 22–39.
3. Schour, I., Tweedy, W. R., and McJunkin, F. A. The effect of single and multiple doses of the parathyroid hormone on the calcification of the dentin of the rat incisor. *Am. J. Path.*, 1934, **10**, 321–342.
4. Von Spreter, T. Experimentelle Untersuchungen über die Wirkung des bestrahlten Ergosterins auf die parathyreoprive Ratte. *Ztschr. f. d. ges. exper. Med.*, 1932, **85**, 19–45.
5. Schour, I., Chandler, S. B., and Tweedy, W. R. Changes in the teeth following parathyroidectomy. I. The effects of different periods of survival, fasting, and repeated pregnancies and lactations on the incisor of the rat. *Am. J. Path.*, 1937, **13**, 945–970.
6. Taylor, N. B., Weld, C. B., Branion, H. D., and Kay, H. D. A study of the action of irradiated ergosterol and of its relationship to parathyroid function. *Canada M. A. J.*, 1931, **24**, 763–777.
7. Jones, James H. The effect of the administration of cod liver oil upon thyroparathyroidectomized dogs. *J. Biol. Chem.*, 1926, **70**, 647–657.
8. Hess, A. F., Weinstock, M., and Rivkin, H. A further report on the effect of thyroparathyroidectomy on action of irradiated ergosterol. *Proc. Soc. Exper. Biol. & Med.*, 1929–30, **27**, 298–299.

9. Shelling, David H. Effect of viosterol on serum calcium of parathyroidectomized rats. *Proc. Soc. Exper. Biol. & Med.*, 1930–31, **28**, 303–305.

10. Tweedy, Wilbur R., McNamara, E. W., Templeton, R. D., and Patras, Mary C. The effect of calciferol on the serum calcium level in the nephrectomized and in the thyroparathyroidectomized-nephrectomized rat. *J. Biol. Chem.*, 1937, **119**, p. xcix.

11. Erdheim, J. Über die Dentinverkalkung im Nagezahn bei der Epithelkörperchentransplantation. *Frankfurt. Ztschr. f. Path.*, 1911, **7**, 295–342.

DESCRIPTION OF PLATE

PLATE 144

Fig. 1. Microphotograph of a longitudinal section of the upper left incisor of Rat 29 which lived 102 days after parathyroidectomy and was given 1 injection of 25 (Collip) units of parathyroid extract per 100 gm. of body weight 4 days before death. The tooth outline is slightly irregular. Note the vascular inclusions and the irregular stratification of the dentin. The injection effect is found near the pulp and is more prominent in the lingual dentin. Al. b. = alveolar bone; En. sp. = enamel space left by the decalcification process of enamel; L. a. p. = labial alveolar periosteum; Od. = odontoblasts. Compare with Figures 2 and 4. × 5.25.

Fig. 2. Microphotograph of the proximal third of the lingual dentin indicated in Figure 1. D. po. = dentin laid down previous to replacement therapy; D′ = dentin laid down and calcified after the injection showing modified and improved calcification. This dentin (D′) is 66 μ wide, corresponding with the 4 day survival period after the injection (4 × 16). Cem. = cementum; Od. = odontoblasts; P. d. m. = periodontal membrane; Pre. = predentin. × 45.5.

Fig. 3. Microphotograph of a longitudinal section from the labial dentin of the upper right incisor of Rat 67 which lived 39 days after parathyroidectomy and was given 1 injection of 78 (Collip) units of parathyroid extract 9 days before death. Note the characteristic parathyroprivic dentin (D. po.) and the more normal calcification following replacement therapy. En. sp. = enamel space; D. m. = mantle dentin; D′ = dentin formed and calcified after injection; Pre. = predentin; Od. = odontoblasts. × 120.

Fig. 4. Microphotograph from the middle third of the labial dentin indicated in Figure 1. L. a. p. = labial alveolar periosteum; En. ep. = enamel epithelium. Note its disturbance at X. En. sp. = enamel space showing persistence of organic enamel matrix. D. po. = dentin laid down before replacement therapy showing interglobular dentin and indications of vascular inclusions; D′ = dentin laid down and calcified after the injection showing modified calcification and 65 μ wide. This amount corresponds closely to the survival period after the injection; Od. = odontoblasts; Pre. = predentin. × 45.5.

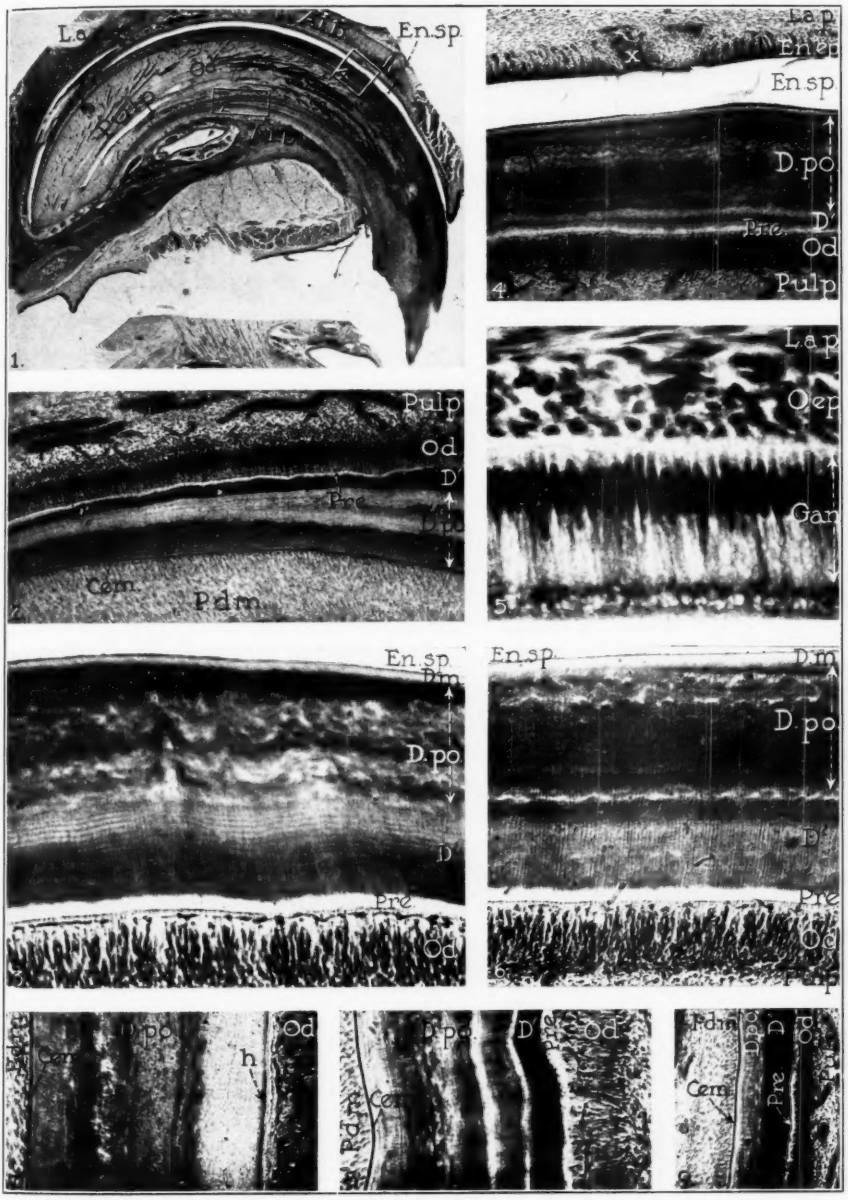
Fig. 5. Microphotograph of a longitudinal section from the proximal enamel epithelium of the upper incisor of Rat 38 which lived 20 days after parathyroidectomy and was given 1 injection of 14.7 (Collip) units of parathyroid extract 3 days before death. Note the globules at the distal end of the ganoblasts. Gan. = ganoblasts; L. a. p. = labial alveolar periosteum; O. e. p. = outer enamel epithelium. × 420.

Fig. 6. Microphotograph of a longitudinal section from the midregion of the labial dentin of the upper left incisor of Rat 111 which lived 146 days after parathyroidectomy. It received 2 injections of parathyroid extract 3 days apart. The last injection was given 7 days before death. D. po. = dentin laid down previous to replacement therapy; D′ = dentin laid down and calcified after the injection. This dentin shows a more homogeneous calcification. En. sp. = enamel space; D. m. = mantle dentin; Od. = odontoblasts; Pre. = predentin. × 100.

Fig. 7. Microphotograph of a longitudinal section from the midregion of the lingual dentin of the lower right incisor of Rat 113 which lived 121 days after parathyroidectomy and was given 1 administration of 552,000 international units of calciferol 24 hours before death. D. po. = dentin laid down previous to the administration. Note the alternate zones of various degrees of calcification disturbances, particularly the uncalcified zone formed preceding the time of administration. Note the very narrow hematoxylin staining line (h) immediately next to the narrow predentin. This line was produced by the administration of calciferol and corresponds in position to the time of administration of the calciferol. Cem. = cementum; Od. = odontoblasts; P. d. m. = periodontal membrane. × 100.

Fig. 8. Microphotograph of a longitudinal section from the midregion of the lingual dentin of the upper incisor of Rat 17A which lived 60 days after parathyroidectomy and was given 184,000 international units of calciferol 96 hours before death. D. po. = dentin laid down before administration of calciferol. Note its alternate zones of varying degree of disturbances in calcification. D′ = dentin laid down and calcified after the administration. This dentin is 68 μ wide, corresponding with the 96 hour survival period after the administration of calciferol. Cem. = cementum; Od. = odontoblasts; P. d. m. = periodontal membrane; Pre. = predentin. × 87.5.

Fig. 9. Microphotograph of a longitudinal section from the proximal third of the lingual dentin of the upper left incisor of Rat 6A which lived 62 days after parathyroidectomy and was given 460,000 international units of calciferol 144 hours before death. D. po. = dentin laid down previous to the administration. D′ = dentin laid down and calcified after the administration of calciferol. This dentin is 103 μ wide and corresponds closely to the 144 hour survival period after the administration. Cem. = cementum; Od. = odontoblasts; P. d. m. = periodontal membrane; Pre. = predentin. × 44.5.



Schour, Tweedy, Chandler, Engel

Changes in Teeth Following Parathyroidectomy. II.

MORPHOLOGICAL CHANGES IN THE PITUITARIES OF RATS RESULTING FROM COMBINED THYROIDECTOMY AND GONADECTOMY *

ISOLDE T. ZECKWER, M.D.

(From the Department of Pathology, University of Pennsylvania, School of Medicine, Philadelphia, Pa.)

The recent physiological developments in our knowledge of the reciprocal relations between the pituitary and other endocrine organs need correlation with the morphological changes associated with altered secretory phenomena. Just as the many studies made of the primary lesions of the pituitary affecting other organs of internal secretion prove to be of great practical and clinical importance, so do changes reflected in the pituitary when the peripheral endocrine organs have been altered furnish data of theoretical significance.

"Castration cells" which develop in the pituitary of the rat after removal of the gonads are well known. Less well known are the "thyroidectomy cells" which appear in the pituitaries of all animals. As some of the literature on the nature of these cells has been reviewed in a recent study of such cells,[1] it need not be reviewed here. There has been considerable difference of opinion as to whether these cells are chromophobes or basophiles, and whether they are identical with castration cells or not. In a study of 9 male thyroidectomized rats Sevringhaus and coworkers [2] report that after thyroidectomy in the rat the basophile increase gives "to the pituitary the castrate appearance," that "large numbers of typical castration cells are present," and that "basophiles of the thyroidectomized rats are similar to those of castrate and thyroid-treated rats."

It was thought that a study of the pituitary after thyroidectomy and gonadectomy had been performed at the same time would be a means of determining whether or not thyroidectomy cells are identical with castration cells. The results obtained would have a functional significance, which will be discussed later. In previous reports [3, 4] it was stated that thyroidectomy cells can be distinguished from castration cells, but histological descriptions of changes in the pituitary following combined thyroidectomy and

* Aided in part by a grant from the National Research Council.
Received for publication June 21, 1937.

gonadectomy have not been published by the author. These changes were described orally, however, at the meeting of the American Association of Pathologists and Bacteriologists in 1936.

Methods

The pituitaries of 32 male and 30 female white rats which were thyroidectomized and gonadectomized at the same time (35 to 40 days of age) were studied. The rats were killed from 44 to 171 days after operation. In some animals one adrenal was removed as well. The pituitaries and other organs were weighed and then fixed in Helly's fluid and stained with Mallory's aniline blue stain for connective tissue.

Results

Histological Changes: The pituitaries of rats that had been submitted to this combined operation were compared with those of over 200 rats that had been merely thyroidectomized, and with those of 29 rats that had been merely castrated. On each microscopic slide were mounted sections of several pituitaries of rats subjected to different types of operations, so that the staining technique would be the same for the group. This procedure makes patent the difference in staining properties between thyroidectomy and castration cells. Furthermore, in the pituitaries of rats with combined operations the distinction is just as clear. The neglect to control staining by mounting on the same slide experimental and control pituitaries probably accounts for many of the discrepancies found in the literature.

In the pituitaries from rats that had a combined operation there are sometimes considerably more thyroidectomy cells than castration cells. The castration cells show deep blue staining coarse granules, whereas the thyroidectomy cells show very fine granules of the size of those seen in chief cells. The hyaline material in the castration cells is usually clearly demarcated from the cell granules, giving a signet ring appearance; whereas the hyaline material in the thyroidectomy cells appears denser in character and involves the entire cell. The thyroidectomy granules are interspersed through the hyalin in several portions of the cell while one or more portions of the cells are free of granules. The junction, therefore, of hyalin and cell granules is a very irregular line.

After thyroidectomy alone, the loss of acidophiles is striking. In certain pituitaries almost every acidophile has disappeared, in others less extensive loss has occurred but conspicuous degranulation of acidophiles is seen. This loss of acidophiles is associated with visceral dwarfing. By looking at the pituitary microscopically one can estimate roughly the degree of retardation of kidney growth, which is corroborated by the weights recorded in the protocols. This is consistent with the generally accepted view that acidophiles elaborate growth hormone, and seems to me to account for the dwarfing of cretins. These experiments have dealt chiefly with thyroidectomy at an early age, but in the few large rats that were thyroidectomized the kidney weights were less than in the litter-mate control, as though even in the adult, maintenance of visceral weights is dependent on acidophiles. The production of thyroidectomy cells seems to occur at the expense of the acidophiles, which are seen in various stages of degranulation, while castration cells are produced without making any demands on the acidophiles. It is as though the cells had a different source and development.

In the earlier experiments dealing with simple thyroidectomy, the thyroidectomy cells seemed definitely basophilic cells, though staining with more difficulty than ordinary basophiles. Since studying pituitaries of rats that had combined operations, the staining properties of the cells could be contrasted with greater detail. In these the impression was gained that the blueness of the thyroidectomy cells was due more to the intracellular hyaline material which forms a diffuse background in which the cell granules appear suspended than to the staining properties of the granules themselves. This may account for the difference of opinion as to whether the thyroidectomy cells are basophiles or chief cells. It may also explain why the descriptions of pituitaries from human cretins and thyroidectomized rabbits do not seem consistent with the findings in the pituitaries of thyroidectomized rats, cats and dogs. For instance, Bryant,[5] and Marine, Rosen and Spark,[6] do not describe hyalin-containing basophilic cells in the thyroidectomized rabbit pituitary, but rather a hypertrophy of chief cells associated with the loss of acidophiles. In a thyroidectomized rabbit pituitary I have studied, cells of the same character as the thyroidectomy cells of rats, cats and dogs have been found, except

that the intracellular hyalin was small in amount and stained inconspicuously so that the blueness of the cell was merely a tinging and not so apparent.* There may then be species differences in the formation of the hyaline material and it may be that the staining of the hyalin contributes more to the blueness of the cell than does the staining of the granules. These cells are abnormal cells whatever their classification may be.

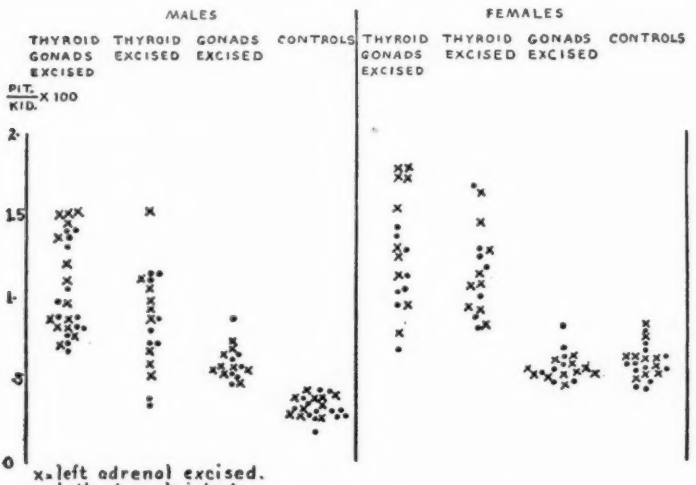


CHART 1

The pituitaries of rats after combined operations leave no doubt that the thyroidectomy cells are totally different from castration cells, whether or not their blueness should permit them to be called basophiles.

No recognized change was produced by unilateral adrenalectomy.

Pituitary Weights: The weights of rats' pituitaries after thyroidectomy, gonadectomy, and combined thyroidectomy and gonadectomy, were compared in a larger series of rats than those included in the histological study. The highest absolute weights

* With hematoxylin and eosin staining the peculiar character of thyroidectomy cells cannot be recognized in any species. These cells require special staining.

were obtained in the combined group notwithstanding the marked dwarfing of the animals. In spite of the loss of acidophiles, the increased numbers of abnormal cells and intracellular hyalin had greatly increased the size and weight of the pituitary. Grossly the pituitary was tense, rounded and engorged, in contrast with the pale, flat, flaccid pituitary of normal rats. In Chart 1 only rats that were operated on at 5 weeks and killed at 11 weeks of age are recorded. Unilateral adrenalectomy did not alter the weight. The use of absolute weights in comparing the pituitaries of animals of greatly varying size is confusing. The percentage of pituitary weight to body weight is fallacious because the somatic subcutaneous tissues are greatly thickened in both the stunted thyroidectomized rat and the overly large castrated rat. Therefore, the ratio of pituitary to kidney weight is used in the chart in order to contrast pituitary growth with visceral growth. In terms of absolute weights, the mean weights of pituitaries are shown in Table I.

TABLE I

	Control	Gonadectomized	Thyroidectomized	Thyroidectomized and gonadectomized
Males	6.3 (mean of 23 rats)	10.7 (mean of 16 rats)	9.3 (mean of 18 rats)	13.0 (mean of 27 rats)
Females	9.7 (mean of 22 rats)	8.0 (mean of 24 rats)	12.3 (mean of 16 rats)	13.5 (mean of 18 rats)

DISCUSSION

Considering the fact that there are many secretory principles elaborated by the pituitary and yet only three recognized histological types of cells, several explanations may be considered as possibilities. A cell of given accepted histological type may be secreting more than one hormone, or a given activator principle may have several effects, depending on the peripheral endocrine organ, or we are not recognizing all the histological types of pituitary cells that really exist.

When a peripheral endocrine organ is diseased or ablated, presumably the pituitary cell that reacts in consequence is the cell producing the hormone that affects the peripheral end organ in question. For instance, after castration of rats, certain basophiles seem to be storing within their cytoplasm an excess of secretion,

and it has been shown that such castration pituitaries contain an excess of gonadotropic hormone (Evans and Simpson [7]) and they are discharging into the blood stream an increased amount of gonadotropic hormone. A reasonable explanation for this is that when the internal secretion produced by the peripheral end organ no longer is present in usual amounts, there is compensatory hyperactivity of the pituitary in forming the hormone which stimulates that peripheral endocrine organ. But when there is no end organ to act on, the pituitary secretion which ordinarily stimulates that peripheral organ is accumulating unused in the cell. Similarly, after thyroidectomy certain cells show accumulation of hyaline material which has the appearance of stored secretion, and the pituitary from stunted thyroidectomized rats contains an abundance of thyrotropic hormone.[8] The thyroidectomy cells probably are the cells producing the thyrotropic hormone. Since the present experiments indicate that thyroidectomy cells are distinct from castration cells, these two histological types of cells probably produce different hormones.

In the normal pituitary, basophiles vary in their degree of staining and in the size of their granules. This is generally interpreted as corresponding to phases of secretion and discharge of secretion, the coarsely granular being regarded as "ripe." It is altogether possible, however, that these different blue staining cells really represent cells producing different hormones. That is, that under normal conditions we are not capable of differentiating different types of basophiles, but when the pituitary is altered by thyroidectomy, gonadectomy, or combined thyroidectomy and gonadectomy, then these blue staining cells are dissociated and their histological differences are appreciated.

Summary

When thyroidectomy and gonadectomy are carried out at the same time in rats, greater hypertrophy of the pituitary occurs than after thyroidectomy alone or after gonadectomy alone.

After the combined operation both thyroidectomy cells and castration cells appear in the same pituitary.

Thyroidectomy cells are distinctly different from castration cells in many histological characteristics, and the two cells probably produce different hormones.

After the combined operation acidophiles degranulate and largely disappear, just as after thyroidectomy alone. The loss of acidophiles is associated with retardation in visceral growth.

REFERENCES

1. Zeckwer, Isolde T., Davison, Lewis W., Keller, Thomas B., and Livingood, Clarence S.II. The pituitary in experimental cretinism. I. Structural changes in the pituitaries of thyroidectomized rats. *Am. J. M. Sc.*, 1935, **190**, 145–157.
2. Sevringhaus, A. S., Smelser, George K., and Clark, Helen M. Anterior pituitary changes in the adult male rat following thyroidectomy. *Proc. Soc. Exper. Biol. & Med.*, 1933–34, **31**, 1127–1129.
3. Zeckwer, Isolde T. The adrenals and gonads of rats, following thyroidectomy, considered in relation to pituitary histology. *Am. J. Physiol.*, 1936, **116**, 166.
4. Zeckwer, Isolde T. The adrenals of rats after thyroidectomy and gonadectomy, considered in relation to pituitary histology. *Am. J. M. Sc.*, 1937, **194**, 145.
5. Bryant, Arthur R. The effect of total thyroidectomy on the structure of the pituitary gland in the rabbit. *Anat. Record*, 1930, **47**, 131–143.
6. Marine, David, Rosen, S. H., and Spark, Charles. Effect of iodine and desiccated thyroid on anterior pituitary of goitrous and thyroidectomized rabbits. *Proc. Soc. Exper. Biol. & Med.*, 1934–35, **32**, 803–810.
7. Evans, Herbert M., and Simpson, Miriam E. Comparison of anterior hypophyseal implants from normal and gonadectomized animals with reference to their capacity to stimulate the immature ovary. *Am. J. Physiol.*, 1929, **89**, 371–374.
8. Zeckwer, Isolde T. Thyrotropic effect of pituitaries from cretin rats. *Am. J. Physiol.*, 1936, **117**, 518–524.

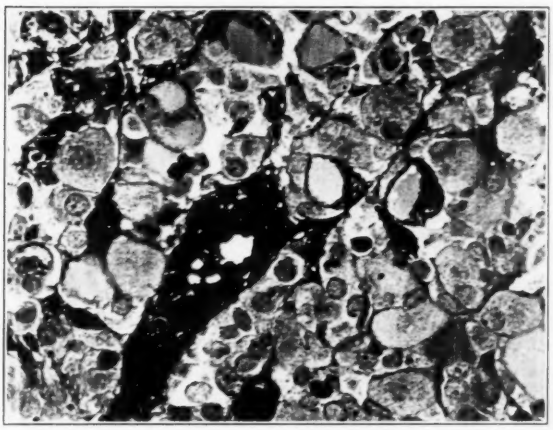
DESCRIPTION OF PLATE

PLATE 145

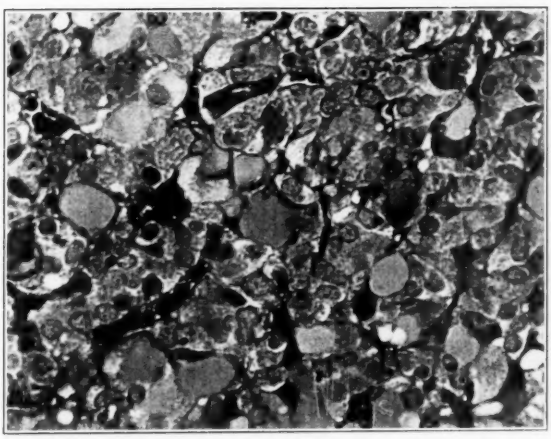
FIG. 1. Rat 9–D–4, female, 16 weeks of age, 11 weeks after thyroidectomy, gonadectomy, and unilateral adrenalectomy. Two castration cells are seen to the right of the upper end of the large blood vessel. All other hyalin-containing cells are thyroidectomy cells. × 566.

FIG. 2. Rat 9–E–1, male, 16 weeks of age, 11 weeks after thyroidectomy and gonadectomy. Two castration cells are seen in the upper right hand corner. All other hyalin-containing cells in this field are thyroidectomy cells. × 566.

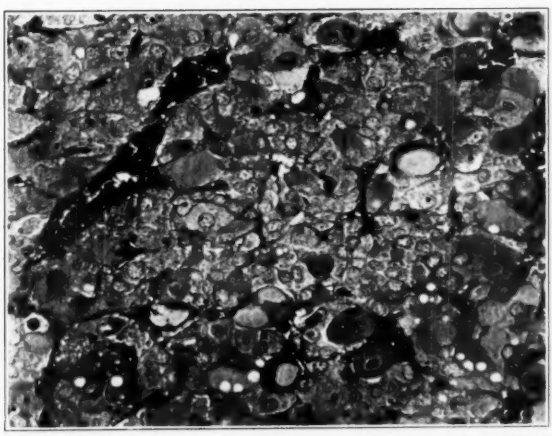
FIG. 3. Rat 10–A–4, male, 16 weeks of age, 11 weeks after thyroidectomy, gonadectomy, and unilateral adrenalectomy. A castration cell is seen in the middle of the lower border, and one at the right a little above the middle. Nearly all the other hyalin-containing cells are thyroidectomy cells. × 393.



1



2



3

Zeckwer Morphological Changes in Pituitaries of Rats

SILVER IMPREGNATION OF RETICULUM IN PARAFFIN SECTIONS *

G. GÖMÖRI, M.D.

(From the Third Surgical Unit of the Royal Hungarian Petrus Pázmány University of Budapest, Hungary)

Since Maresch first used the Bielschowsky silver stain for the demonstration of connective tissue fibrils, and especially since its results in paraffin sections proved to be at least as reliable if not better than those obtainable with frozen material, the Bielschowsky-Maresch silver impregnation has become one of the most widely used special staining methods. Although many connective tissue stains of different types have been devised since, silver impregnation still ranks first because of its sharp delineation of the finest fibrils. Like all important methods it too has many modifications, each claiming some superiority to the original technique. As I was unable to find data in the literature concerning the relative merits and reliability of the various modifications, and, secondly, as the rôle and importance of the individual steps of the impregnation process are almost unknown, I decided to investigate this problem systematically. For the time being I used formalin-fixed material only, embedded in paraffin, or in celloidin-paraffin according to the rapid method of Erös. It is my plan also, however, to extend further my investigations concerning the action of the fixatives. My results to date are as follows: First, I was impressed by the occasional high degree of similarity, almost identity, of the results obtained by methods seemingly most dissimilar. On the other hand, there is no known method, the results of which would be as reliable and constant as those of, for instance, the hematoxylin nuclear stain, all silver methods being liable to yield more or less variable pictures. Even unexplainable complete failures are by no means rare. There is, however, a great difference in the reliability index of the various methods, some of them being notoriously prone to complete failure, and even in the event of success yielding pictures of variable quality; whereas with other methods failure is most exceptional and the results are remarkably uniform. A second essential difference between the various methods lies in the different amount of reticular meshwork demon-

* Received for publication July 6, 1937.

strated. Some methods almost invariably reveal a decidedly smaller number of fibrils than are demonstrated by other methods. If one happens to be accustomed to use routinely one of the methods demonstrating a relatively sparsely woven reticulum, he may be convinced, by lack of comparison, of the effectiveness of his method and quite unaware of the fact that other methods demonstrate a much richer fibrillar structure than the method he adopted. He probably would be surprised if informed that he never had seen a really complete fibrillar picture.

The successive steps of the silver impregnation method are discussed below and it is hoped that the information given will be of value to the various workers interested in the demonstration and study of reticulum.

1. *Oxidation-Reduction:* Oxidation of the sections with potassium permanganate, followed by reduction with oxalic or hydrobromic acid, or, according to my experience, even better with acid potassium sulphite (so-called metabisulphite), is an essential part of the procedure, greatly reducing the number of failures and ensuring more uniform results. Wilder suggests the use of a 10 per cent phosphomolybdic acid solution instead. I, too, obtained many beautiful preparations with this latter method, but its reliability being decidedly inferior to that of the permanganate treatment, the routine use of the latter is more recommendable. The undesirable action of the permanganate treatment, consisting of loosening of the sections on the slide and often their actual floating away in the further course of impregnation, can easily be obviated by a suitable technique to be described later. According to Foot, the permanganate is liable to impair nuclear staining; therefore he suggests the use of a pyridine-glycerin mixture instead. I have never observed impairment of nuclear staining caused by permanganate treatment. Moreover, I found the effect of the pyridine-glycerin mixture to be much inferior to that of permanganate oxidation and by no means suitable to replace it. The mechanism of action of the permanganate solution is not well understood. That it is not due to mere oxidation has been proved by Foot. He was unable to obtain the same effect with any other oxidizing agent. I repeated his experiments and tested a few additional substances, such as sodium perborate, ammonium persulphate, chromic acid and a compound solution of iodine. The results were

identical. I also concur in Foot's finding that both manganese salts and oxalic acid in themselves are inert.

2. *Sensitization:* I wish to apply this term to any treatment of the section with metal salts or other substances preceding the use of the ammoniacal silver solution. Suitable sensitization also is an important step, causing the fibrillar structure to become more completely visualized. Tannic acid and certain metal salts are sensitizing agents. The tannic acid treatment seems to be inferior to that of the metal salts as it is less reliable and the ground substance is likely to become dark brown, greatly impairing the clarity of the picture. Hence, metal salts are to be preferred. I assayed the salts of the following metals: aluminum, silver (used in the original method of Bielschowsky), gold, cadmium, chromium, cobalt, copper, iron (ferrous and ferric), mercury, magnesium, manganese, nickel, lead, stibium, tin, uranium (suggested by Wilder) and zinc. Marked and uniform sensitization was obtained with silver, gold, cadmium, ferric, lead, tin and uranium salts. The richest reticulum is obtained with iron sensitization. The sensitizing action of the other metals is either nil, slight, inconstant, or not selective. Gold, lead and tin salts often cause undesirable precipitates, though otherwise the reticular structure is excellent. There remain silver, cadmium, ferric and uranium compounds. As mentioned, silver sensitization is a part of the original Bielschowsky method and of certain of its modifications. Originally the use of a 2 per cent solution of silver nitrate for 24 to 48 hours was recommended. I found that exactly the same effect can be obtained within 2 minutes if a 10 per cent solution is used. In this way time can be saved. The original method warns against a more than superficial rinsing of the sections in distilled water after sensitization, as longer rinsing is liable to weaken the stain. This I cannot confirm. On the contrary, I strongly advise thorough washing of the sections in several changes of distilled water. This slight modification will result in distinctly clearer pictures, better nuclear staining, and complete absence of precipitates. The same technique applies to all other metal sensitizations (cadmium, uranium, and especially iron). I used the nitrates of cadmium and uranium in 1 to 2 per cent solutions. The duration of sensitization is about 1 minute; longer exposure does not enhance the effect nor does the combined use of several metal salts. The ferric com-

pound I used was iron ammonium sulphate, the same substance used in Heidenhain's iron hematoxylin stain. I employed freshly prepared 1 to 2 per cent solutions. The time of exposure should be 1 minute. The peculiarity of iron sensitization is the brilliant metachromasia obtained on gold toning.

3. *Silver Impregnation:* Most modifications concern the preparation of the ammoniacal silver solution. In general, three different types of solutions are used:

1. From silver nitrate silver hydroxide is precipitated with sodium or potassium hydroxide and the precipitate is dissolved in ammonia.

2. From silver nitrate silver carbonate is precipitated by some soluble carbonate and the precipitate is dissolved in ammonia.

3. To the silver nitrate solution ammonia is added drop by drop, until the precipitate which forms on addition of the first few drops is again dissolved.

There are many formulas for the preparation of the solutions, some of which are characterized by almost extravagant accuracy — for instance, the formulas of Kubie and Davidson. I started my experiments with solutions of the Type 1 (silver-ammonia hydroxide). I varied the amount of added alkali from one-half to three volumes. The only difference I noticed was the proportionately quicker action of the more alkaline solutions. However, the final results obtained with the different solutions were extremely similar, indeed identical to such extent that I would have been unable to distinguish the sections stained with the different solutions had I not marked them beforehand. Therefore, in my opinion, too great accuracy in preparing the ammoniacal silver solution is entirely superfluous. Of course, it is better not to use too strongly alkaline solutions as they are liable to damage the sections. Solutions prepared with one-half to three-fourths equivalent amount of alkali are the most suitable, *i.e.* to one volume of 10 per cent silver nitrate solution one-sixth to one-fourth volume of a 10 per cent solution of potassium hydroxide is added. The same applies to carbonate solutions. The amount of ammonia seems to be more important. According to most formulas even a slight excess of ammonia is likely to produce inferior results, especially if the hydroxide type of solution is used. According to my experience there is a certain optimal amount of ammonia; both more or less will produce unsatisfactory results. If there is an excess

of ammonia the picture will be very sharp and distinct, but a part of the fibers will escape impregnation; whereas if too little ammonia is used the ground substance will be dark and the picture blurred. There are different methods for securing the right amount of ammonia, the simplest of which are the following: to the precipitate ammonia is added drop by drop, while the container is continuously shaken, until the last grains are just dissolved and then either (1) silver nitrate is again added cautiously until it is easily dissolved on stirring the solution, or (2) the vessel containing the solution is placed in hot water until black silver precipitate begins to form on its surface. Solutions prepared in either way can be used for 2 or 3 days if kept in stoppered bottles. The silver precipitate that collects on the bottom of the bottle does not interfere with the staining capacity of the solution. Solutions of the carbonate type and those prepared with ammonia only, keep well for at least 5 to 6 days. Solutions of the hydroxide type are to be diluted with distilled water to twice their volume and used at room temperature. The time of exposure is about 1 to 3 minutes. If cadmium, iron or uranium sensitization is used, 1 minute will suffice and the sections will show almost no change in color; whereas if silver sensitization is used it is better to prolong impregnation to 3 minutes, until the sections become pale tobacco brown. Solutions of Types 1 and 2 stain only at higher temperatures (37 to 50° C.) and should be diluted to 4 to 5 times their volume before use. When comparing the different solutions I found that those of the hydroxide type are the most reliable and yield the most uniform results; whereas with the carbonate solution and with the solution prepared with ammonia only, failures are not uncommon. However, in the case of success the silver carbonate stain excels in producing absolutely even, delicately shaded pictures, free of precipitate. All solutions stain the cells also. At times excellent nuclear staining is obtained, on other occasions the cytoplasm will be stained. Very often different parts of the same section show different cellular staining. The cause of this phenomenon is unknown. In general, with cadmium and uranium sensitization the chromatin pattern is more distinct than if iron or silver is used. After impregnation the sections are washed in distilled water for 5 to 10 seconds. Longer exposure to distilled water weakens the stain.

4. *Reduction:* Formalin is used for this purpose, the concentra-

tion of which within wide limits does not appreciably influence the result. In contrast with the findings of Foot I found the reaction of formalin unimportant. Simple commercial formalin, neutralized, slightly alkalinized or acidulated solutions, gave identical results. The duration of reduction should be at least 3 minutes. After reduction the sections are washed in running water.

5. *Gold Toning:* Successful toning produces beautiful shades. The reticulum is dark black, collagen fibers are rose to brick red, nuclei rusty brown to deep red. Unfortunately, it is not always possible to produce this range of shades. The cause of occasional failures is unknown. However, there are several factors decidedly enhancing metachromasia. These are iron sensitization, prolongation of gold toning to at least 10 minutes, and finally the reduction of the toning with oxalic acid (according to Laidlaw), or even better with potassium metabisulphite. The action of the latter compound is instantaneous. By employing this combination failures can be prevented with almost absolute certainty.

6. *Fixation:* Fix in a 1 to 2 per cent solution of sodium thiosulphate (hyposulphite) for 1 minute. Longer fixation will impair the distinctness of the finest fibers. After fixation the sections are thoroughly washed in running water, then treated with 2 changes of alcohol, cleared with xylol and mounted in balsam. Foot suggested counterstaining of the sections with hematoxylin and picro-fuchsin. In my opinion this counterstaining is unnecessary; moreover, the fact that after the van Gieson stain it is often impossible to determine whether certain fibers are stained by fuchsin or by gold, outweighs its possible advantages.

In summarizing my results, I may say that all methods omitting either permanganate oxidation or sensitization, or both, are decidedly unreliable and the reticulum picture they yield is, even in the case of success, incomplete. Far the best sensitizing agent I have tried is iron ammonium sulphate.

I wish now to describe my own modification of the Bielschowsky-Maresch reticulum impregnation which gave complete satisfaction in a series of several hundreds of sections. The only material I had failures with is bone marrow, especially the fatty type, whereas highly cellular marrow, as seen in leukemia and in some cases of pernicious anemia, gave beautiful pictures. The poor impregnability of bone marrow is well known to all who have

tried to study its reticulum by means of silver impregnation, and it is mentioned also by Orsós. After having tried all methods described I am convinced that no method is certain of reliability in this respect.

My modification is as follows:

Run paraffin sections through xylol, then 2 changes of alcohol and wash under the tap.

1. Oxidize with a 0.5 to 1 per cent solution of potassium permanganate for 1 to 2 minutes. Rinse in tap water.

2. Decolorize with a 1 to 3 per cent solution of potassium metabisulphite for 1 minute. Wash under the tap for several minutes.

3. Sensitize in a 2 per cent solution of iron ammonium sulphate (violet crystals) in distilled water for 1 minute. Wash under the tap for a few minutes, then run through 2 changes of distilled water.

4. Impregnate with the following solution for 1 minute:

To a 10 per cent silver nitrate solution add one-sixth to one-fourth its volume of a 10 per cent solution of potassium hydroxide. Add strong ammonia water drop by drop, while shaking the container continuously, until the precipitate is completely dissolved. Add again, cautiously, silver nitrate solution drop by drop until the resulting precipitate easily disappears on shaking the solution. Make up the solution with distilled water to twice its volume. It can be kept in a stoppered bottle for 2 days.

5. Rinse quickly in distilled water for 5 to 10 seconds.

6. Reduce for 3 minutes in commercial formalin diluted with tap water to 5 to 10 times its volume. Wash under the tap for a few minutes.

7. Tone in a 0.1 to 0.2 per cent solution of gold chloride for 10 minutes. Rinse in distilled water.

8. Reduce toning in a 1 to 3 per cent solution of potassium metabisulphite for 1 minute.

9. Fix in a 1 to 2 per cent solution of sodium thiosulphate (hyposulphite) for 1 minute.

Wash under the tap. Run through alcohol of increasing percentages. Clear in xylol and mount in balsam.

As mentioned before, paraffin sections occasionally will float away during impregnation with the strongly alkaline silver solu-

tion. This annoyance can be easily prevented by affixing the sections to the slide with gelatin instead of egg albumin-glycerin. The gelatin must be subsequently hardened by formalin fumes. The method is as follows: Dilute the glycerin-gelatin mixture commonly used for fluid preservation of sections with water or glycerin until it remains fluid at room temperature. Spread a thin layer of this solution on the slide and affix sections. Dry the slides in the incubator at 37° C. in formalin fumes for at least 10 hours. (Pour commercial concentrated formalin into an open Petri dish and place it in the incubator.) The formalin has to be removed from the sections as even traces of it will inhibit impregnation. This is easily accomplished by exposing the slides in a similar manner to the action of ammonia vapor for several hours.

BIBLIOGRAPHY

Erös, G. Rasches Verfahren zur Herstellung doppelt eingebetteter Schnitte. *Centralbl. f. allg. Pathol. u. path. Anat.*, 1925–26, **36**, 249–251.

Foot, Nathan Chandler. A technic for demonstrating reticulum fibers in Zenker-fixed paraffin sections. *J. Lab. & Clin. Med.*, 1923–24, **9**, 777–781.

Foot, N. C., and Ménard, M. C. A rapid method for the silver impregnation of reticulum. *Arch. Path. & Lab. Med.*, 1927, **4**, 211–214.

Foot, Nathan Chandler. Chemical contrasts between collagenous and reticular connective tissue. *Am. J. Path.*, 1928, **4**, 525–544.

Foot, N. C., and Day, H. A. The occurrence of reticulum in tumors. *Am. J. Path.*, 1925, **1**, 431–443.

Foot, N. C. Notes on the rapid impregnation of reticular tissue with silver. *J. Tech. Methods*, 1929, **12**, 117–119.

Foot, Nathan Chandler, and Foot, Ellen Bellows. A technique of silver impregnation for general laboratory purposes. *Am. J. Path.*, 1932, **8**, 245–254.

Gorriz, Mariano. Quelques observations sur la composition des bains d'argent dans les méthodes de Bielschowsky et leurs variantes, et résultants du procédé de Bielschowsky-Cajal appliqué à l'histopathologie. *Trav. du lab. de recherches biol. de l'Univ. de Madrid*, 1924, **22**, 169–190.

Kubie, Lawrence S., and Davidson, David. The ammoniacal silver solutions used in neuropathology: their staining properties, chemistry and methods of preparation. *Arch. Neurol. & Psychiat.*, 1928, **19**, 888–903.

Laidlaw, George F. Silver staining of the skin and of its tumors. *Am. J. Path.*, 1929, **5**, 239–247.

Maresch, Rudolph. Ueber Gitterfasern der Leber und die Verwendbarkeit der Methode Bielschowskys zur Darstellung feinster Bindegewebsfibrillen. *Centralbl. f. allg. Pathol. u. path. Anat.*, 1905, **16**, 641–649.

Orsós, F. Das Bindegewebsgerüst des Knochenmarks im normalen und pathologischen Zustand. *Beitr. z. path. Anat. u. z. allg. Pathol.*, 1926, **76**, 36–86.

Pap, T. Eine neue Methode zur Imprägnation des Retikulums. *Centralbl. f. allg. Pathol. u. path. Anat.*, 1930, **47**, 116.

Schlemmer, A. Ueber die Herstellung der ammoniakalischen Silbersalzlösung bei der Imprägnationsmethode von Bielschowsky. *Ztschr. f. wissensch. Mikr.*, 1910, **27**, 22.

Wilder, Helenor Campbell. An improved technique for silver impregnation of reticulum fibers. *Am. J. Path.*, 1935, **11**, 817–819.

DESCRIPTION OF PLATES

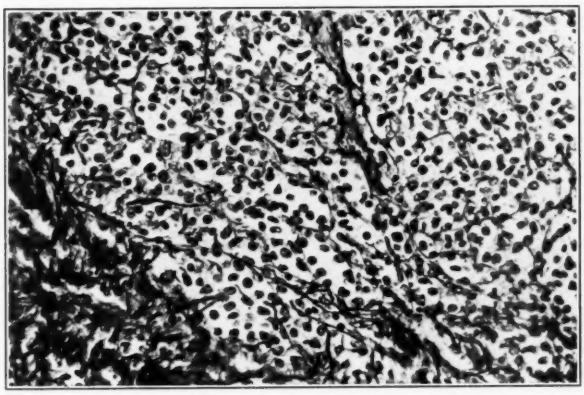
Microphotographs of Figs. 1 to 5 show corresponding fields of serial sections of a block from a case of subcutaneous round cell sarcoma. Figures 6 to 9 show corresponding fields of serial sections from a leukemic spleen. (The distention of the vascular spaces is artificial and was produced by the injection of formalin solution into the splenic vessels.) All microphotographs have been made under strictly identical optical conditions.

PLATE 146

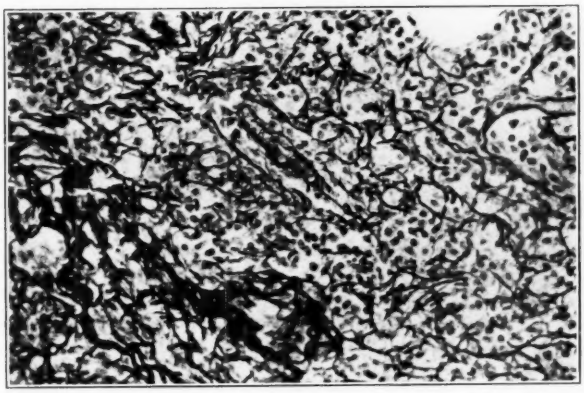
FIG. 1. Foot's stain, Variant II.

FIG. 2. Silver sensitization (author's modification).

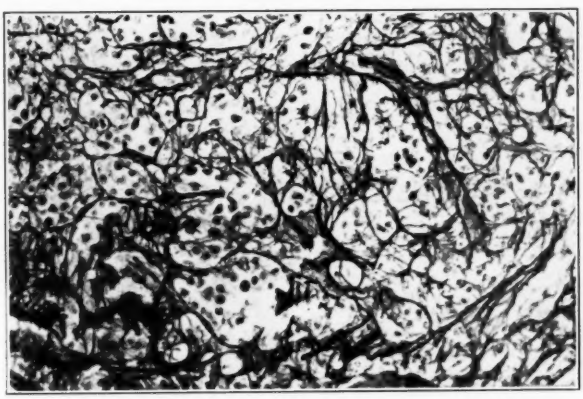
FIG. 3. Uranium sensitization (method of Wilder).



1



2



3

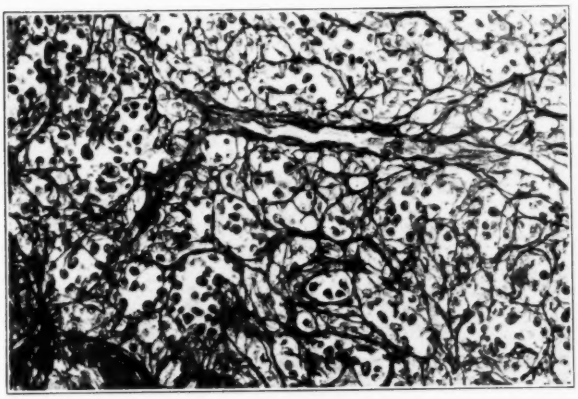
Gömöri Silver Impregnation of Reticulum

PLATE 147

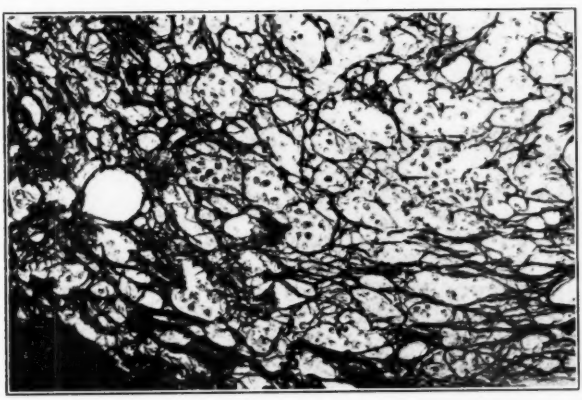
FIG. 4. Cadmium sensitization.

FIG. 5. Iron sensitization.

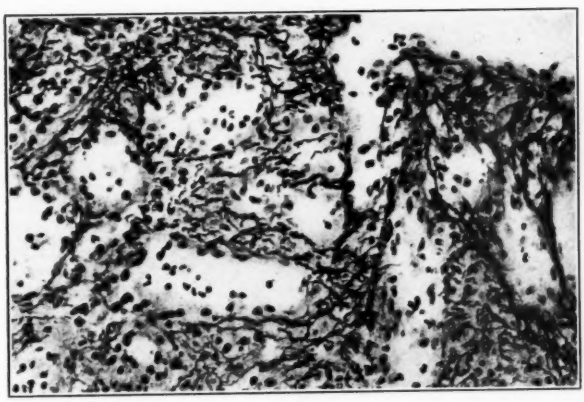
FIG. 6. Foot's stain, Variant II.



4



5



6

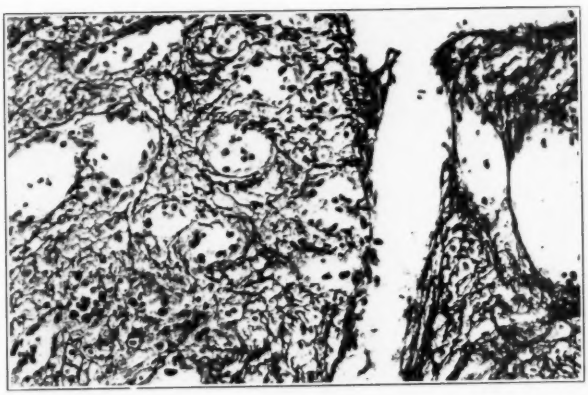
Gömöri Silver Impregnation of Reticulum

PLATE 148

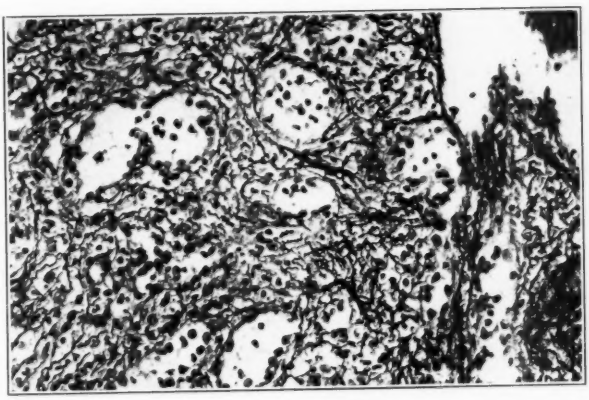
FIG. 7. Uranium sensitization (method of Wilder).

FIG. 8. Cadmium sensitization.

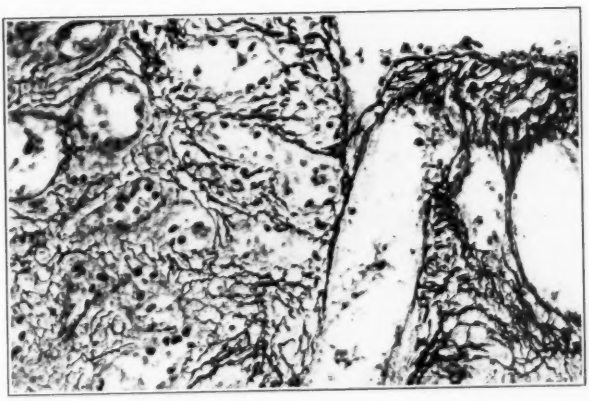
FIG. 9. Iron sensitization.



7



8



9

Gömöri Silver Impregnation of Reticulum

CEREBRAL MEDULLOBLASTOMA *

WILLIAM MURRAY HONEYMAN, M.B., CH.B.†

(From the Department of Pathology, College of Physicians and Surgeons, Columbia University, and the Department of Neuropathology, Neurological Institute, New York, N. Y.)

The occurrence of primary medulloblastoma in the cerebrum deserves record on account of its rarity. The question has been raised as to whether it exists at all. Therefore, it must be considered whether theories of the origin of medulloblastoma in the cerebellum preclude its occurrence in the cerebrum, and proof must be advanced that the tumor is identical when it arises in the cerebrum.

Bailey and Cushing [1] considered the medulloblast a bipotential cell, which might give rise equally to glial elements and nerve cells. They accepted the hypothesis of Schaper that the cells of the external granular layer, earlier recognized by Hess as a transitory structure, made up a secondary germinal layer and acted as a depot of indifferent constituents able to develop neuroblasts and spongioblasts. They considered medulloblastoma to have its origin in retained rests of embryonic cells of this kind. Accordingly medulloblastomatous tumors might be of a primitive cell type or vary considerably in their growth and degree of departure from the primitive cell form. Thus in his atlas Bailey [2] portrayed medulloblastomas with a preponderance of cells with spherical vesicular nuclei, each with a heavy nucleolus better identified with special silver stains. These he considered neuroblasts. On the other hand, a medulloblastoma might show a fair proportion of spongioblastic elements.

The view of the French school is rather different. To Roussy and Oberling the constituents of the medulloblastoma of Bailey and Cushing are not indifferent cells. They find a preponderance of neuroblastic cells. Considering the similarity of the elements of the embryonic medullary tube, the "neurosponge," they would designate the tumor neurospongioma. They admit that these tumors are not made up of pure growths of a cell type stopped at one precise point in cell evolution. For that reason they would

* Received for publication June 22, 1937.

† Commonwealth Fellow.

reject other terms that have been suggested — neurocytoma, neuroblastoma and neurogliocytoma.

There is much to suggest the origin of medulloblastoma in embryonic rests, apart from the morphological characteristics recalling a type cell found in the embryo. The majority give rise to symptoms early in life, and occur in the midline where cell rests are more frequent. Brody and German [3] found cell rests in the region of the posterior medullary velum in 75 out of 400 supposedly normal cerebellums. Wohlwill [4] noted the roof of the fourth ventricle as a favorite site of rests. Commenting on the persistence of germinal clusters in the first month of extrauterine life, he agreed with Wanke [5] that these cells were related to unripe glial elements and neuroblasts. He considered some of the cells more mature neuroblasts. The occurrence of medulloblastomas in identical twins observed by Cushing [6] also gives support to the theory of origin in cell rests.

Stevenson and Echlin [7] have recently suggested that medulloblastoma arises from a specific cerebellar structure. They describe a number of cases of tumor apparently arising from the external granular layer of the cerebellum and suggest the name granuloblastoma. Thereby, they wish to designate a tumor of a cell destined to give a special type of nerve cell — the granule cell. They argue that if the basic Schaper theory be true, then a definitely gliomatous tumor might be expected on occasion. With Schaper, Ostertag,[8] Jacob [9, 10] and Hayashi [11] agree that the external granular layer not only gives rise to the broader molecular layer with nerve cells, the elements of the inner granular layer, but is also a new source of quite indifferent cells for the cerebellum as much for the ganglion cells as for the glial elements. They draw attention to the numerous cell elements migrating from the external granular layer to the inner, elements they consider bipotential. While a specific granule cell stain is wanting, there can be no absolute indication that the external layer forms only granule cells. A theory of origin of medulloblastoma from a specific cerebellar structure must exclude the acceptance of cerebral medulloblastomas.

On the other hand, the theory of origin in cell rests may also be applied to a consideration of medulloblastoma of the cerebrum, for embryonic rests are known to occur there. Jacob [9] describes the broadening of the white matter from the 5th month of fetal life.

Everywhere remnants of the intermediate layer appear as connected bands or as cell tubes and islands. Isolated groups of these embryonal cells may persist for some time. Such heterotopias, surviving from the invasive and migratory phase of cerebral cellular development, have been the subject of many reports. Since they often occur in relation to a blood vessel, although eccentric, they have been misinterpreted as encephalitis congenita.[12] Naturally they have been more frequently observed in the brains of premature infants. Ferraro and Barrera[13] described a remarkable case of megalo-myelo-encephaly with diffuse medulloblastosis in a child of 12 years. There was evidence of retained embryonic development with the added element of neoplasia. A suggested explanation was that cells which had not reached their ultimate destination in the central nervous system had maintained their embryonic activity; at points in the cerebrum apparently indifferent cells had gone on to form elements of both the spongioblastic and neuroblastic series — a neoplastic process recalling tuberosclerosis. There may be in the cerebrum, therefore, sources of embryonic cells such as those that account for the appearance of medulloblastoma in the cerebellum.

The following 2 cases show that tumors morphologically identical with the infratentorial medulloblastomas occur also in the cerebrum.

Case Reports

Case 1. *Clinical History:* Male, aged 36 years. For about 1 year following death of the patient's mother from glioblastoma multiforme, the history was one of personality changes. Difficulty in walking and dizziness preceded by 14 days an increasing left hemiplegia with stupor. There was no other complaint and physical examination revealed no abnormalities save the neurological. By roentgenogram the pineal was found displaced posteriorly and to the left, indicating an expanding lesion within the right cerebral hemisphere. Ventriculogram revealed displacement of the lateral and third ventricles to the left; the right temporal horn was not visualized. At operation a large infiltrating mass presented high up in the temporal lobe and a second mass in the posterior part of the frontal lobe. Much tumor was removed but complete eradication was not possible. Small cystic collections of yellow fluid were encountered.

A course of X-ray treatment was given. Power in the left limbs improved for some 6 weeks. With assistance the patient was able to walk. He remained facetious and overactive mentally. Thereafter deterioration was rapid. Homonymous hemianopsia, blurring of disc margins, paresis and hypesthesia of the left side and general hyperreflexia developed. The patient died 12 weeks from the time of operation.

Pathological Findings

Autopsy was limited to the head. On removal the cerebral hemispheres were found asymmetrical, the gyri flattened and sulci narrowed over the convexity, especially on the right side. In the right temporal region was the raised zone of operative trauma. The chiasm, basal ganglia and midbrain were compressed by the grossly enlarged temporal lobe. The rest of the brain stem and the cerebellum showed no abnormalities.

Section showed in the entire right occipital lobe and in the posterior part of the parietal lobe, a granular, cream colored tumor with indefinite edges and yellowish degenerated and hemorrhagic areas. It measured approximately 3 by 3 by 3.5 cm. It extended to the white matter of the right temporal lobe where there was a large cystic zone, the site of operation. The cyst communicated with the inferior horn of the lateral ventricle. It measured 6 cm. in length by 5.5 and 3.5 cm. The ependymal lining of the ventricles was smooth; the right lateral ventricle was narrowed, and the left dilated.

Microscopic Examination

The tumor extensively invaded the central white matter and cortex. It consisted of cells fairly densely packed save in the central areas of necrosis and in the periphery where columns were especially arranged along the blood vessels which were numerous and showed considerable endothelial hyperplasia. A pseudorosette arrangement was sometimes observed, tumor cells being gathered in rings with the tail of pyriform protoplasm directed towards the center. The cells were mostly round, some polygonal and a few more pyriform. The amount of cytoplasm was generally small. Exceptional cells showed a larger amount, but in it no granules, vacuoles, blepharoplasts or Nissl substance were found. The nuclei were round or oval and their chromatin arranged in deeply staining masses. Infrequently a definite nucleolus was found. The variation in the diameter of the nucleus was represented graphically. In general like limits of variation were found in different parts of the tumor. Local collections of larger or small nuclear types were rarely encountered. The impression was that at no special points was there deviation towards a different cell type. Division by mitosis and amitosis was frequent, with preponderance

of the latter method. Around the blood vessels large vacuolated mononuclears were found and in areas of operation and irradiation there was degeneration with the presence of numerous lymphocytes, phagocytic monocytes and considerable budding of the capillaries and endothelial proliferation. There, too, free adventitial cells occurred and required careful differentiation from atypical tumor cells which might possibly be undergoing change to the spongioblastic series. However, with Cajal's stain no true unipolar or bipolar spongioblasts were shown. While demonstrating a few fibrous astrocytes related to the persisting structure of the invaded parenchyma, it failed to reveal in the tumor cells an affinity for gold. In central areas where tumor cells were densely packed and where the misleading appearance of invaded or degenerated brain substance was lacking, cells taking the gold sublimate stain did not occur. To this evidence that the tumor cells were undifferentiated was added a test with Cajal's reduced silver stain for neurofibrils. This failed to show either neurofibril formation even in the more elongated and tail-like types of cytoplasmic process, or the local deposition of silver at one extremity of the cytoplasm (fibrillogenous zone of Held) which has been taken by some authors as evidence of the differentiation of a neuroblast.

Examination of the contralateral hemisphere, the brain stem and cerebellum failed to show any further extension of the tumor.

CASE 2. *Clinical History:* Female, aged 3 years. The child was a full term baby delivered normally. Development appeared usual, the child walking and talking at the average age.

In July, 1933, the patient fell and struck her head. There was no loss of consciousness. In September she had severe frontal headaches, and in November she developed tremor and stiffness in the left leg so that it was usually maintained in the extended position. About the same time the mother noticed some rigidity on lifting the child and since then weakness of the left leg increased and the child became unable to walk. Vomiting occurred occasionally; it was not projectile and occurred after taking food. There was incontinence of urine. Tremor of the right hand at rest was noted a fortnight before admission in May, 1934.

Examination in the Neurological Institute showed moderate rigidity in extension, particularly in the left leg. Significant findings were Magnus-de Kleijn reflexes, hyperreflexia, especially in the lower limbs, absence of abdominal reflexes, dilated pupils, the left failing to react to light, optic atrophy of left disc, thought to be due to preceding papilledema, and blurring of the right disc margins. Plantar responses were not elicited. The cerebrospinal fluid was yellow, with 6 cells, 508 mg. protein, 63 mg. sugar, and 693 mg. chlorides per 100 cc. fluid. The Wassermann reaction on the fluid was negative

and the colloidal gold curve 111000000. Roentgenograph of the skull showed an area of scattered calcification to the right of the midline, measuring 0.5 by 3 cm. on anterior posterior view, and 4.5 by 2 cm. in the lateral. The flecks of calcification formed an arch roughly paralleling the contour of the lateral ventricle. The impression was one of intraventricular tumor or deep parieto-temporal glioma.

Fits with generalized tremors, profuse sweating, rotary nystagmus on looking to the left, and hyperpyrexia preceded death which came 5 days after admission to the hospital.

Pathological Findings

Complete autopsy was performed. The calvarium was thin with some separation of the sutures, the dura tense, the leptomeninges thin and transparent. The brain weighed 1040 gm. The cerebral hemispheres were asymmetrical; flattening of the gyri was general but most marked in the anterior part of the greatly enlarged right temporal lobe. Herniation through the incisura tentorii cerebelli accounted for deep indentation of the hippocampal and fusiform gyri on the right side, and for displacement of the infundibulum, optic chiasm and cerebral peduncles to the left. From the widened posterior end of the sulcus olfactorius protruded a round, cream colored, slightly lobulated mass, 2 cm. in diameter. The floor of the third ventricle was herniated downwards.

Section of the cerebellum showed no abnormality. Section of the brain stem showed concave indentation of the midbrain in the right side, the substantia nigra and red nucleus being especially compressed. The pons was distorted and the floor of the fourth ventricle showed marked granular ependymitis.

The wide extent of the tumor on the right side from frontal to occipital lobes was shown on section of cerebrum. It involved almost all the temporal lobe and the white matter of the frontal, parietal and occipital lobes. It invaded deeply the caudate nucleus, putamen, globus pallidus and thalamus, and it grew down into the orbital gyri. It was roughly 9.5 cm. in length and 5 cm. at its greatest diameter. The margins were indistinct. The tumor was cream colored, firm and granular except in scattered areas of necrosis and hemorrhage. In the parietal region within the tumor was a large cyst with firm but ragged walls. The left lateral ventricle was compressed and like the right and third ventricle showed granular ependymitis with occasional larger nodules representing tumor.

There were no findings of note in the trunk organs.

Microscopic Examination

The tumor in the right hemisphere consisted of densely packed cells with numerous blood vessels and rare areas of necrosis. The cells were frequently arranged in clusters and in the pseudorosette formations of Wright. At the periphery, and especially where invading the white matter, they were found in long columns. Even in these areas the polygonal or round shape of the scanty cytoplasm persisted. Where the cells were more discrete and free from compression, the cytoplasm showed a fluffy outline or tail-like process, such as is regularly associated with the cells of cerebellar medulloblastoma. Calcium occurred in small amounts irregularly, free and in relation to the vessel walls.

The spherical or rather oval shape of the nuclei was defined by a distinct and thick nuclear membrane. They stained deeply and showed a coarse chromatin network. Mitoses were numerous. The variation in size of nuclei is shown graphically in Figure 1. The cell bodies did not stain with Mallory's phosphotungstic acid hematoxylin, though rare blue staining fibers were found in the tumor. They most likely represented glial fibers of the invaded brain tissue.

By Cajal's gold sublimate stain a number of hypertrophied fibrous astrocytes were shown in the periphery of the tumor between the tumor cells. They were considered to belong to the invaded parenchyma. No elongated immature cells of the spongioblastic or astroblastic series were revealed, nor were neuroblastic forms detected with a silver neurofibril stain.

The wall of the left lateral ventricle was stripped of ependymal cells and there was proliferation of subependymal astrocytes. A large flat tumor mass of essentially the same type as that in the right cerebral hemisphere represented a tumor implant.

Section of midbrain showed marked compression of the substantia nigra, cerebral peduncles and, to a lesser extent, the red nuclei. Ependymal granulations in the wall of the aqueduct of Sylvius partially occluded the lumen at one point. Gliosis and thickening of the external glial membrane were present. The cerebellum and roof of the fourth ventricle were free of tumor growth.

Discussion

The diagnosis of medulloblastoma in both cases rests on the general features of the cells, round, oval or pyriform with scanty

cytoplasm and well stained nucleus, their fairly uniform size and their arrangement in compact masses, sometimes with pseudo-rosette formation. The perinuclear halo and polygonal shape of the cells of oligodendroglioma were absent. Special stains for oligodendroglia with silver failed but while they are notoriously difficult in tumor tissue, further proof that oligodendroglial cells were not the constituent elements came from Mallory's connective tissue stain. Even in parts taken from the central portions of the tumor there was no suggestion of the blue staining of the inter-

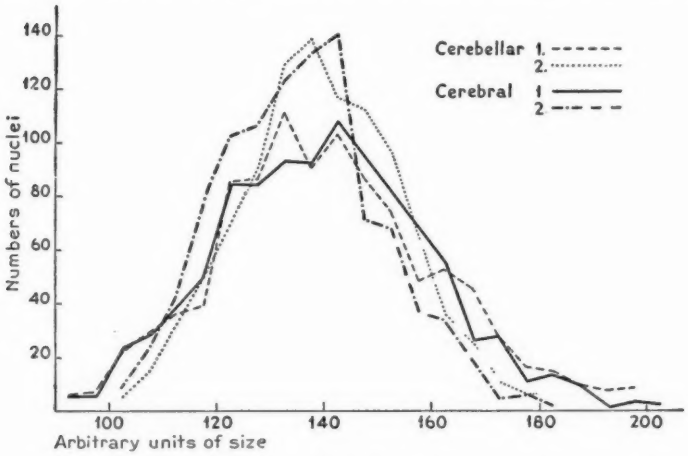


CHART 1

cellular substance such as is found in oligodendroglioma.[14] The tumor cells were unlike those of ependymoma. The absence of true rosettes and the failure of Bailey's neutral ethyl violet-orange G to show blepharoplasts also make the diagnosis of ependymoma unlikely.

With phosphotungstic acid hematoxylin and Cajal's gold sublimate, the tumors were shown not to contain spongioblasts or astrocytes and, therefore, cannot be classified with the more mature gliomas. On the other hand, the proof of a neuroblastic evolution is lacking since neurofibril formation was not detected. Thus the diagnosis of a more mature neuroblastic tumor is eliminated.

The cells of these tumors are then essentially of a younger type and without the staining qualities that would indicate differenti-

ation along neuroblastic or spongioblastic lines. In this respect they are similar to cerebellar medulloblastoma of primitive type.

If the origin of cerebral medulloblastoma is comparable with that of the cerebellar, then the tumor might be expected to occur in the sites of election of congenital rests. Amolsch [12] has found that such points are in the angle between the caudate nucleus and

TABLE I

	1	*2*	*3*	*4*
	Cerebral 1	Cerebral 2	Cerebellar 1	Cerebellar 2
Mean nuclear diameter in μ	5.32	5.17	5.30	5.25
S.D.	0.94	0.76	0.42	1.37
P.E.	0.63	0.59	0.28	0.87

the thalamus and at the upper lateral angle of the lateral ventricle in sections through the anterior half of the brain. At term, neurogenic activity is also seen in a few small round cells in the lateral wall of the posterior horn of the ventricle. Owing to the extensive spread in the cases here described it is impossible to determine the point of origin of the tumor.

The similarity of the cerebral to the cerebellar medulloblastomas is also seen in the distribution of nuclear sizes figured in Chart 1. Micrometer measurements of 1000 nuclear diameters were

TABLE II

	1 & 2	*1 & 3*	*1 & 4*	*2 & 3*	*2 & 4*	*3 & 4*
$\frac{\text{Mean Diff.}}{\text{P.E. Diff.}}$	0.29	0.30	0.10	0.05	0.09	0.06

made in each of the cerebral tumors and in primitive type cell medulloblastomas of the cerebellum picked at random from the files. The conditions of fixation and staining were strictly similar. From the mean, standard deviation and probable error of these measurements (Table I) the ratio $\frac{\text{mean difference}}{\text{P. E. difference}}$ was figured (Table II). It gives an index from which one may judge whether the nuclear size distributions are essentially comparable or not.

In the comparison of any two tumors it does not exceed three. The conclusion rests therefore that the constituents of the tumors do not differ in nuclear size significantly.

Since some investigators have described neuroblasts in medulloblastic tumors, our 2 cases were compared with the cerebellar medulloblastomas of primitive type in order to find any differences in this respect. The absence of neurofibril formation has been noted. Other criteria of neuroblastic evolution have been considered. Bailey [2] has suggested that the normal growth of a neuroblast from a less differentiated cell is first shown by swelling of the nucleus. If a similar evolution were taking place in the tumor cells, then possibly the curve of distribution of nuclear sizes in a cerebral medulloblastoma might be biphasic or show a marked shift to the right on comparison with that of medulloblastoma cerebelli of primitive type. No suggestion of this is seen. While the tables serve to indicate that the range of variation is not greater than that which may occur in medulloblastoma cerebelli, that the predominant nuclear sizes of this tumor above and below the cerebrum are the same and the tumors are alike, no further conclusions can be drawn. Heiberg [15] has rightly directed attention to the variation in size and shape of the nucleus in malignant tumors. This consideration must limit the inferences to be drawn from measurements of nuclei in neoplasms.

Conspicuous nucleoli were occasionally found in the cells of both the cerebral and cerebellar medulloblastomas. They have been taken by some authors as a sign of differentiation of a neuroblast. As early as 1896 Pianese [16] noted changes in the nucleolus of cancer cells; today a study of nucleoli is a valuable aid in the diagnosis of malignant tumor, for the nucleolar nucleus ratio is increased considerably. Whether large nucleoli are related to rapid growth or whether the changes are only features of atypical cell proliferation is quite uncertain; yet surely enlargement cannot be taken as an additional diagnostic characteristic of a specific cell type.

It might be suggested that these tumors are made up of neuroblasts which have not yet reached the stage of formation of Nissl substance and neurofibrils. It may be that a number of the cells are of such a type and that primitive cells, sprung from the medullary epithelium and morphologically alike, have determining factors for

one specific evolution. Yet in the absence of characteristics of development of a special type, it is impossible to do more than conjecture in what direction cells of this primitive type are tending. In any case the evidence of evolution is as much lacking for these cerebral as for some cerebellar medulloblastomas. Our studies have shown no essential difference. The histological diagnosis of a primitive cell tumor is also consistent with the life history in the cerebral cases. Cushing has emphasized that it is insufficient to define the microscopic character of a tumor. For each there is a more or less typical life history to be correlated. Here in two cerebral medulloblastomas there is a comparatively rapid course, which contrasts with the history of the majority of Cushing's cases. In 1930 in a review of medulloblastoma Cushing [6] stressed the later age incidence, the slower growth and the more favorable outlook in those of the cerebrum. These features he associated with a more differentiated type of cell. He classified his cases as medulloblastoma, medulloblastoma neuromatosum, and neuroblastoma, according to the degree of deviation from the neuroblastic type. The latter showed least malignant features. As lengthy a preoperative history as 8 years and a 5 year survival after operation were recorded. His cases of tumor of the simple true medulloblastomatous type in the cerebrum were in males aged 37 years and 9 years. They gave histories of 3 years and 4 months and they survived operation for periods of 3 years 4 months and 2 months respectively. In the first of our cases, that of a male, 35 years of age, the history of tumor rapidly proliferating and accounting for death despite operation and irradiation 14 months from onset is without doubt the story of a tumor of a high degree of malignancy. In the second, the early incidence at the age of 3 years and the short course recall typical features of cerebellar medulloblastoma away from the midline. On clinical as well as on histological grounds the diagnosis of undifferentiated cerebral medulloblastoma seems warranted.

Summary

The origin of medulloblastoma in the cerebrum is discussed and the 2 cases described are shown to be essentially identical with the cerebellar medulloblastomas in point of morphology and life history.

REFERENCES

1. Bailey, Percival, and Cushing, Harvey. A Classification of the Tumors of the Glioma Group on a Histogenetic Basis with a Correlated Study of Prognosis. J. B. Lippincott Company, Philadelphia, 1926, 9.
2. Bailey, Percival. Histologic atlas of the gliomas. *Arch. Path. & Exper. Med.*, 1927, **4**, 871–921.
3. Brody, B. S., and German, W. J. Medulloblastoma of the cerebellum: report of 15 cases. *Yale J. Biol. & Med.*, 1933, **6**, 19–29.
4. Wohlwill, F. Zur Frage der sogenannten Encephalitis congenita (Virchow). *Ztschr. f. d. ges. Neurol. u. Psychiat.*, 1921, **68**, 384.
5. Wanke, R. Zur Anatomie und Chirurgie des Medulloblastoms. *Arch. f. klin. Chir.*, 1933, **177**, 528–564.
6. Cushing, H. Experiences with the cerebellar medulloblastomas; critical review. *Acta path. et microbiol. Scandinav.*, 1930, **7**, 1–86.
7. Stevenson, Lewis, and Echlin, Francis. Nature and origin of some tumors of the cerebellum: medulloblastoma. *Arch. Neurol. & Psychiat.*, 1934, **31**, 93–109.
8. Ostertag, B. Die typischen Geschwulstbildungen des Kleinhirns und ihre Beziehungen zu den Fehlbildungen des übrigen Medullarrohres. *Klin. Wchnschr.*, 1932, **11**, 1651.
9. Jacob, Alfons. Normale und pathologische Anatomie und Histologie des Grosshirns. Deuticke, Vienna, 1927, **1**, 71.
10. Jacob, A. Nervengewebe. Handbuch der mikroskopischen Anatomie des Menschen, W. von Möllendorff, Ed. J. Springer, Berlin, 1928, **4**, Pt. 1, 755.
11. Hayashi. (Quoted by Jacob.)
12. Amolsch, Arthur L. The interpretation of the histologic findings in encephalitis congenita. *Am. J. Clin. Path.*, 1935, **5**, 371–383.
13. Ferraro, A., and Barrera, S. E. Megalo-myelo-encephaly: report of a case with diffuse medulloblastosis. *Am. J. Psychiat.*, 1935, **92**, 509–526.
14. Greenfield, J. Godwin, and Robertson, E. Graeme. Cystic oligodendrogliomas of the cerebral hemispheres and ventricular oligodendrogliomas. *Brain*, 1933, **56**, 247–264.
15. Heiberg, K. A. Die Grundlage der Geschwulstlehre. Kabitsch, Leipzig, 1933.
16. Pianese, G. Beitrag zur Histologie und Aetiologie des Carcinoms. G. Fischer, Jena, 1896.

DESCRIPTION OF PLATE

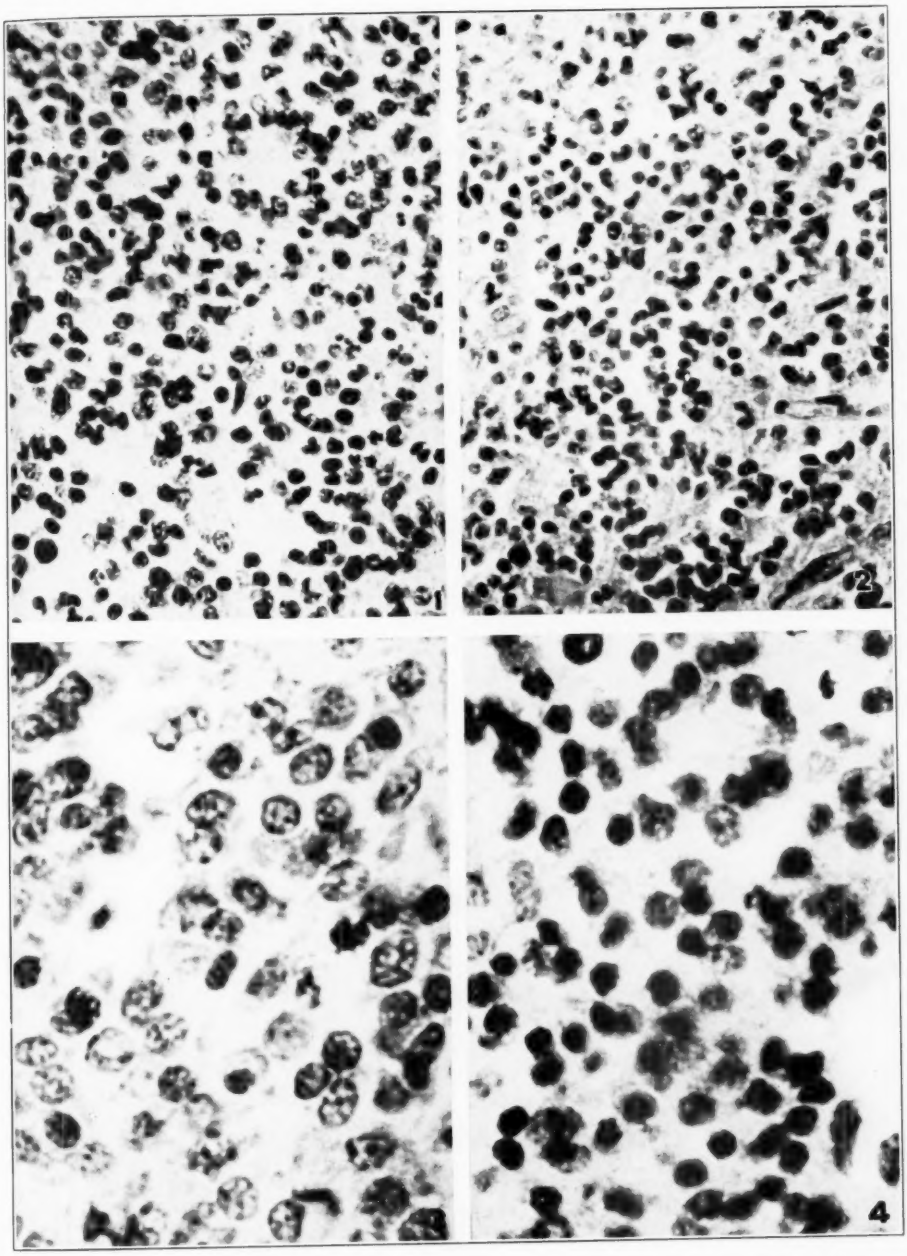
PLATE 149

FIG. 1. Case 1. Showing uniform distribution of cells and pseudorosettes. Hematoxylin-eosin stain. × 600.

FIG. 2. Case 2. Showing uniform distribution of cells and pseudorosettes. Hematoxylin-eosin stain. × 600.

FIG. 3. Case 1. Cells uniform in size with scanty cytoplasm. Hematoxylin-eosin stain. × 1200.

FIG. 4. Case 2. Showing similar features of cell body and nucleus. Hematoxylin-eosin stain. × 900.



Honeyman

Cerebral Medulloblastoma

INDEX OF SUBJECTS

INDEX OF SUBJECTS

A

B

*Abstract of paper presented at the meeting of the American Association of Pathologists and Bacteriologists held at Chicago, March 25 and 26, 1937.

C

D

E

R

S

T

INDEX OF AUTHORS

INDEX OF AUTHORS

*Abstract of paper presented at the meeting of the American Association of Pathologists and Bacteriologists held at Chicago, March 25 and 26, 1937.

G

H

N

O

P

Q

R

S

VOLUME XIII, No. 6 WHOLE NO. 79

NOVEMBER, 1937

THE AMERICAN JOURNAL OF PATHOLOGY

Official Publication of
The American Association of Pathologists and Bacteriologists

Editorial and Publication Office
818 HARRISON AVENUE, BOSTON, MASSACHUSETTS
Issued Bimonthly Annual Subscription in U. S. A. $8.00

Entered as second class matter, March 20, 1914, at the Post Office at Boston, Massachusetts, under the Act of March 3, 1879

THE AMERICAN JOURNAL OF PATHOLOGY

is owned and controlled by the American Association of Pathologists and Bacteriologists and is edited by a board appointed by the Council of the Association. It is devoted to the prompt publication of original observations and investigations in the field of pathology, especially morphological pathology, and will be published bimonthly to form one volume of at least six hundred pages a year.

Papers will be accepted for publication on condition that they are contributed solely to *The American Journal of Pathology*. Manuscripts must be typewritten, preferably double spaced, and the original copy submitted.

Papers should, if possible, not exceed 20 printed pages. New matter should be presented in a clear, straightforward manner without elaborate detail. The literature should be summarized as briefly as possible, in paragraphs rather than in pages.

References to the literature should conform to the following order: name of author, title of article, name of periodical, year, volume in arabic, and page (*e.g.*, 1924, 20, 125).

Illustrations in black and white for line or half-tone reproduction will be furnished free in moderate numbers. Pictures in colors are much more expensive and will have to be paid for, in part at least, by the contributors.

Illustrations will not be accepted unless they reach a certain standard of excellence technically and present an attractive appearance. The maximum space available for them on a page measures 8 × 5¼ inches. The prominent lines in them should as a rule run horizontally or vertically, not at various angles. All marking lines and letters should be avoided if possible. If absolutely necessary place them inside the limits of the illustrations or of the total space (8 × 5¼ inches) available.

It is useless to submit photomicrographs for publication unless the fields selected are flat and the details sharp, so that the different types of cells described in the accompanying legends can be clearly and easily distinguished.

Fifty reprints are furnished free to contributors; additional copies may be obtained at cost price.

The subscription price is $8.00 net for each volume in the United States; $8.50 net in Canada; $9.00 net for distribution to foreign countries. Subscriptions will be received only in advance.

Address all communications to

F. B. Mallory, M.D., *Editor-in-Chief*,
818 Harrison Avenue,
Boston, Mass., U.S.A.

CONTENTS

PRINTED AT THE HARVARD UNIVERSITY PRESS, CAMBRIDGE, MASS., U.S.A.